Studies on Thiaminase Bacteria

II. Bacteriological Properties of Thiaminase-II Producing Bacilli Isolated from Sea Sand and Sea Mud.

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(Received November 4, 1968)

In the preceding paper the author[1] reported the isolation from sea sand and sea mud of bacteria which produced extracellular thiaminase. Forty isolates were obtained from 110 samples from 12 spots at the beach of western end of Honshū facing to Suō sea. The samples were collected when tide was low from the shore that became the bottom of shallow when the tide was full.

All of the isolates were spore forming bacilli and could be divided into three groups : The first consisted of aerobic thiaminase-I-, the second of aerobic thiaminase-II- and the third of anaerobic thiaminase-I-producers. Eight isolates belonged to the first, two to the second and thirty to the third group. Of these, isolates belonged to the third group were studied by Hayashi et al.[2] and identified as strains of *Clostridium sporogenes*. The bacteriological properties of the eight isolates which belonged to the first group are being studied in this department. In this paper, the bacteriological characteristics of the isolates belonging to the second group will be presented.

EXPERIMENTAL

Cultures used were those of two isolates described above. They were maintained on a agar slant transferring once a month. Purity and the ability to produce thiaminase-II were frequently checked.

Mehtods used for the study of bacteriological characteristics of cultures were essentially the same as those employed by Gordon and Smith[3] for their studies on spore forming bacteria capable of growth at high temperature and by Smith, Gordon and Clark[4] for their taxonomical studies of aerobic spore forming bacteria. Bacto-peptone (Difco) and Bacto-beef extract (Difco) were used throughout.

The composition of the medium used to check thiaminase activity was as follows: Polypeptone (Takeda), 1 % ; meat extract (Wakō), 0.5 % ; and yeast extract (Daigo), 0.1 %. The medium was adjusted to pH 7.2 and aliquot of

8 ml each was distributed to test tubes and autoclaved. The checking of thiaminase activity was conducted as shown in Table 1 using culture supernatant after 4 days cultivation at 37°C as a crude enzymatic preparation.

Table 1. Test for Thiaminase Activity

Tube No.	Thiamine HCl aq. solution[a]	*M*/15 Phosphate buffer (pH 7.0)	Culture supernatant	Pyridine aq. solution[b]	Water
	ml	ml	ml	ml	ml
1. Control 1 (inactivated enzyme)	1	2	0.5[c]	0	1.5
2. Main 1	1	2	0.5	0	1.5
3. Main 2	1	2	0.5	1	0.5
4. Control 2 (blank)	0	2	0.5	0	2.5

After incubating at 50°C for 60 minutes, reaction was stopped by the addition of 0.1 ml of 5N HCl. For the purpose of converting remaining thiamine to thiochrome, 3 ml of BrCN solution and then 2 ml of 10 % aqueous solution of NaOH was added to all tubes. The BrCN solution was prepared by adding 10 % aqueous solution of KCN to cold saturated aqueous solution of bromine. The resulting thichrome of each tube was measured by a conventional fluorometer. When the recovery of thiamine in tube No. 2 (main 1) was 20 % or less of that in tube No. 1 (inactivated enzyme control), the test was repeated using 1 : 5 dilution of culture fluid and ascertained that the reaction was not accerelated in the presence of pyridine (main 2).

a) 5 μg/ml thiamine HCl solution

b) 10 *M* in final concentration

c) The enzyme was inactivated by heating at 90°C for 20 minutes.

RESULTS

1. **Morphology**: Morphology of two isolates was essentially identical and could be described as follows:

i) Vegetative rods: Young rods 0.4 to 0.6 by 1.5 to 3.0 microns; straight or slightly curved; ends rounded; arranged singly or in pairs, not in long chains. Gram negative or variable. When the cultures became old they were rather pleomorphic and wider rods, up to 1.0 micron in diameter, were frequently observed.

ii) Spores: 1.0 to 1.2 by 2.0 to 2.5 microns; ellipsoidal; central to subterminal. Spore wall, especially of polar parts, thick and stainable. Good sporulation was observed on milk agar (Fig. 1 and 2)

iii) Sporangia: Definitely swollen; spindle-shaped to clavate.

2. **Biological characters**: Growth characteristics on or in various media and biochemical properties of the two isolates were also similar each other.

i) Colonies on nutrient agar composed of 1 % of Polypeptone, 0.5 % of

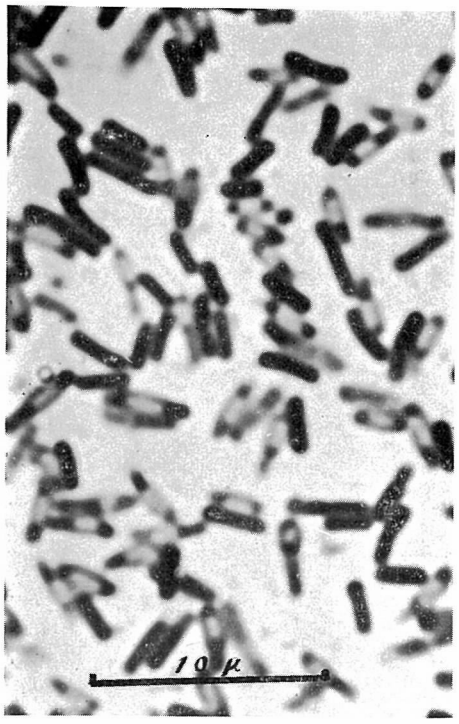

Fig. 1 Isolate No. 1
on milk agar plate

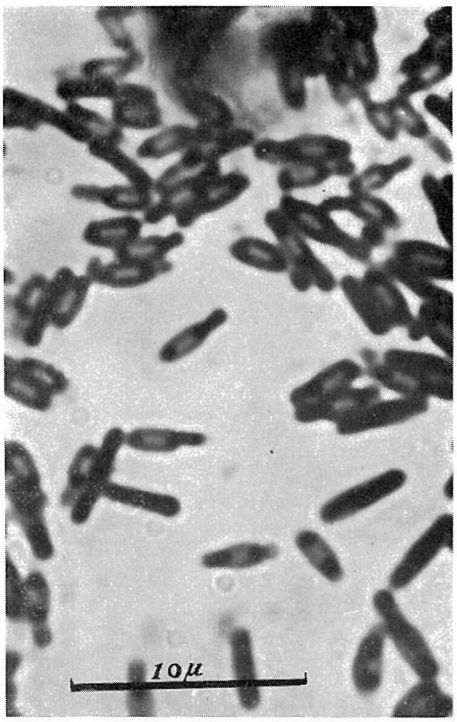

Fig. 2 Isolate No. 2
on milk agar plate

beef extract and 1.8 % of agar: Granular; elebated; became larger and confluent with age; edge crenated.

ii) Nutrient agar slants: Growth thin; translucent; confluent.

iii) Nutrient broth: Turbidity heavy; no pellicle formation observed.

iv) Glucose nutrient agar: Growth slightly more abundant than on nutrient agar.

v) Potato: Creamy growth; potato plugs and the water below the plugs blackend.

vi) Soybean agar slants: Growth better than on nutrient agar slants; dense; spreading.

vii) Anaerobic growth in glucose broth: Negative.

viii) Utilization of citrate: Negative.

ix) Hydrolysis of starch: Negative.

x) Production of acetylmethylcarbinol: Negative.

xi) pH of glucose broth of 7-day-old cultures: 8.4

xii) Hydrolysis of gelatin: Negative.

xiii) Hydrolysis of casein: Negative.

xiv) Production of indole: Negative.

xv) Reduction of nitrate to nitrite: Positive.

xvi) Fermentation tests: Acid without gas from glycerol and fructose. No acid produced from glucose, galactose, mannose, rhamnose, lactose, sucrose, maltose, trehalose, cellobiose, raffinose, arabinose, xylose, dextrin, glycogen, adonitol, mannitol, dulcitol, sorbitol, salicin, esculin and erythritol.

xvii) Thiaminase: Decomposition of thiamine not accelerated by the presence of pyridine.

DISCUSSION

The only species known so far as to produce extracellular thiaminase-II is *Bacillus aneurinolyticus* Kimura and Aoyama.[5] The two isolates studied produced extracellular thiaminase-II, grew well and homogeneously in broth, did not form indole and acetylmethylcarbinol, did not hydrolyze gelatin and starch, reduced nitrates, did not utilize sodium citrate, produced alkali in glucose broth, and produced acid from only a few carbohydrates including glycerol. These observations, together with the morphology of their spores and sporangia, are indicative of their apparent similarity to *Bacillus aneurinolyticus* Kimura and Aoyama, type II.[5]

The "Key to the species of genus *Bacillus*" in Bergey's Manual of Determinative Bacteriology[6] divids species of genus *Bacillus* into three groups. The isolates should belong to the second group of this classification on the morphological basis. The high pH of 7-day-old glucose broth culture and their inability to grow in glucose broth under anaerobic conditions indicate that they related more closely to *Bacillus brevis* than to other members of the group. Because the number of isolates studied was too small and type cultures of *Bacillus aneurinolyticus* and strains of *Bacillus brevis* have not been included in this study, it is difficult to the present author to decide the isolates as belonging to *Bacillus aneurinolyticus* or to aberrant *Bacillus brevis* which is less proteolytic and has lost the ability to produce acid from glucose and sucrose. Whether or not strains of *Bacillus brevis* have the ability to produce thiaminase-II should be investigated in the near future.

SUMMARY

Bacteriological properties of two isolates obtained from sea sand and sea mud were investigated. Although they were similar to *Bacillus aneurinolyticus* Kimura and Aoyama in many points, they also related to *Bacillus brevis*.

REFERENCES

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