STUDIES ON THE AEROBIC MESOPHILIC BACTERIA WITH DISTINCTLY BULGED SPORANGIUM

I. SPECIAL REFERENCE TO *BACILLUS THIAMINOLYTICUS*

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In certain human feces, Chang[1] in 1947 first noted the presence of a thiamin decomposing enzyme, the thiaminase. From such feces and from other subjects, Matsukawa and Misawa[2)3)] isolated many cultures of aerobic sporeforming bacteria, and noted that 1.0 ml of supernate of broth culture of these organisms was able to decompose 1.0 γ of thiamin completely in two hours at 37°C and pH 5. 6. They investigated the cultural characteristcs of six cultures (five from intestine, one from air) of these bacteria, and found that they have the same bacteriological properties.

Fujita and his co-workers[4)5)6)] investigated few strains supplied from D. Matsukawa and reported that they resembled rough strains of *B. alvei* Kimura and his co-workers[7)8)9)] isolated from garden soil a strain of sporulating bacterium having thiaminase activity and compared it with four strains of Matsukawa and Misawa. They reported that these five strains belonged to one species and also noted that they were related to *B. alvei*. They[10)] investigated the thiamin decomposing activity of broth culture of morphologically resembling organisms namely two strains each of *B. circulans*, *B. alvei*, *B. laterosporus* and *B. brevis*, which had been sent from R. E. Gordon and N. R. Smith of the U. S. Department of Agriculture, Belsville, Maryland, but failed to detect any thiamin decomposing activity. Kimura and Aoyama[11)] also isolated 33 similar organisms from 505 patients and healthy subjects in Kyoto-City.

The Members of the Committee of Research of Vitamin B[12)] in Japan agreed to recognize that these bacteria belong to one new species of the Genus *Bacillus* and decided to assign to them the name "*Bacillus thiaminolyticus*".

Later Kimura and Aoyama[12)13)14)] isolated from human feces aerobic sporeforming bacteria having thiaminase activity but with bacteriological characteristics different from *B. thiaminolyticus*. These strains of Kimura and Aoyama were named "*Bacillus aneurinolyticus*" by the Committee.

The morphological and cultural characteristics of *B. thiaminolyticus* have been investigated with only a few strains. Moreover, there are no reports of the presence of such organisms which on account of their morphological and cultural characteristics should belong to *B. thiaminolyticus* but without having any thiaminase activity. We intended to investigate the detailed characteristics of *B.*

thiaminolyticus in comparison with those of morphologically related organisms. Smith[15] revised the description of Genus *Bacillus* in Bergey's Manual of Determinative Bacteriology, sixth edition, 1948. He divided the mesophilic sporeforming aerobes into two groups. The sporangia of the first group are not distinctly bulged, while those of the other are distinctly bulged. He listed *B. alvei* to the latter group and as mentioned above *B. thiaminolyticus* resembles to *B. alvei*. We isolated from feces of healthy human subjects mesophilic aerobic bacteria which formed distinctly bulged sporangia, classified them according to him, and investigated the thiamin decomposing activity of broth cultures.

Media and Methods

1) Media used: The composition of the majority of media used was the same as that used by Gordon and Smith[16] in their studies on aerobic sporeforming bacteria capable of growth at high temperature. Witte peptone was used in nutrient agar, nutrient broth, glucose agar, NaCl broth, peptone acid agar and organic nitrogen base for the fermentation test of carbohydrates. Stock culture agar was prepared according to the original report[17] using Witte peptone and was solidified by 1.5 per cent agar. Glucose was omitted from Löffler's serum. Milk agar plate was prepared by solidifying skimed milk by addition of 15 gm agar per 1,000 ml.

2) Methods for identifying the cultures: They were also similar to those of Gordon and Smith[16]. The incubation temperature for growth was 35°C.

3) For the purpose of indole test, 1.0 per cent Teruuchi peptone (tryptic digest of casein) water of pH 7.0 was used. After 2,3 and 5 days of incubation, the cultures were shaken thoroughly with ether and, after the ether had been collected on the surface, the Ehrlich's reagent was run down the side of the test tube[18]. Reduction of nitrate was tested on a broth culture containing 0.1 per cent KNO_3 grown for 3, 5, and 7 days by the Griess-Ilosva method[19].

4) Thiamin decomposing activity[20)21)]: Fourty eight-hours' and 5 days' broth cultures were centrifuged at 4,000 r. p. m. for 20 minutes, 1.0 ml. of the supernate was mixed with 3.0 ml of 1/15 mol phoshate buffer of pH 6.5 and 1.0 ml of thiamin solution of 1.0 γ per ml. The mixture was incubated in a water bath of 37°C for one hour and the remaining thiamin was titrated with thiochrome reaction using zeorite[22)23)]. When the thiamin in the mixture failed to show the thiochrome reaction, the mixture, after one hour of incubation, was corrected to pH 4.5, heated at 80°C for 15 minutes, and treated at 45°C for one hour with 1.0 ml of 6.0 per cent aqueous extract of Takadiastase whose thiamin had been absorbed by Fuller's earth; if the negative thiochrome reaction had been resulted from phosphorylation of thiamin by aneurinphosphorylase[20)21)], the mixture thus treated would reveal the positive reaction.

5) Isolation of cultures: About one gm of feces was suspended in 10 ml

of saline solution and heated in a water bath of 80°C for 20 minutes. One loopful of this suspension was streaked on nutrient agar plates and another loopful was inoculated into 5 ml of nutrient broth in a test tube. The nutrient broth culture grown at 35°C for 24,48 and 72 hours, was plated on agar. After an incubation of 2,3 and 5 days, searches of colonies on both agar plates was made for bulged sporangia, the isolated colonies representatives of the type and containing bulged sporangia were selected and transferred on agar slants. Colonies which contained no spores in spite of 5-days' incubation were also streaked on agar slants and searched for sporangia after further incubation. Using these methods 135 cultures were collected from 500 healthy human subjects. They were frequently plated on agar and their purity was secured.

For the purpose of comparison 4 cultures of *B. thiaminolyticus* sent from Dr. D. Matsukawa in Niigata University and 10 cultures of *B. thiaminolyticus* and two strains each of *B. circulans* (strains no. 313, 358), *B. alvei* (strains no. 662, 680), *B. laterosporus* (strains no. 882, 1267) and *B. brevis* (strains no. 762, 780) supplied from Prof. R. Kimura in Kyoto University were studied.

Experimental Results

The 135 cultures isolated from feces could be classified as follows: (1) Organisms resembling *Bacillus sphaericus*, 62 strains. (2) Organisms capable of decomposing thiamin by the supernate of their broth culture, 30 cultures. (3). *Bacillus circulans*, 19 strains. (4) *Bacillus alvei*, 6 strains. (5) *Bacillus laterosporus*, 11 strains. (6) *Bacillus brevis*, 1 strain, (7) *Bacillus firmus*, 2 strains. (8) Organisms which could not be identified, 4 cultures.

In this paper the thiamin-decomposing organism isolated is considered to be *B. thiaminolyticus*, studies of the other cultures will be reported later

Bacillus Thiaminolyticus (30 cultures isolated, 14 stock strains received)

Spore: The mature spores were 0.8 to 1.3 by 1.5 to 2.4 μ. They were ellipsoidal and terminal to subterminal. The spore wall was thick and stainable. Remnants of sporangia occasionally were adhesive to matured spores.

Sporangia: In all cultures the sporangia were distinctly swollen terminally, clavate, occasionally spindle shaped. The sporeforming cells appeared after 48-to 72-hours incubation.

Rods: The cells of most cultures measured 0.4 to 1.0 by 2.0 to 5.0 μ, though occasionally long thread-like cells up to 15 μ were recorded. They arranged singly or side by side, but long chain formations could not be observed. All cultures were motile with peritrichous flagella. Rods were Gram-variable; usually Gram-negative, but frequently contained Gram-positive granules.

Optimal temperature for growth: Good growth was obtained between 30 to

37°C on agar slant.

Gelatin liquefaction: On a gelatin stab crateri-form to saccate liquefaction occurred with exception of two strains. When tested by Frazier's technique all strains hydrolyzed gelatin remarkably.

Agar colonies: All cultures possessed more or less spreading abilities. Spreading colonies were translucent and smooth, becoming opaque and corsely granular, the edge of them was undulate but did not resemble that of motile strains of *B. alvei* or *B. circulans*. Colonies of less spreading strains were wrinkled with a lobate edge. Many cultures adhered to the agar

Nutrient broth: Many strains showed an uniform turbidity, becoming clear with thick, somewhat mucoid pellicle. Six strains formed white, thin fragile pellicle but in four strains no pellicle formation was recorded. The supernate after an incubation of 48 hours possessed a remarkable thiaminase activity.

Growth on Witte peptone acid agar, pH 5·0: All cultures were negative.

Growth on soy bean, stock culture and glucose agar: On stock culture agar all strains grow as well as on nutrient agar, but were slightly inhibited on soybean and glucose agar.

Litmus milk and tomato yeast litmus milk: Litmus was reduced by all cultures after a 24-hour incubation. Milk is coagulated after 48 to 72 hours of incubation. Peptonization was observed except for two strains. In litmus milk acid was formed by all cultures but one, though in the studies on fermentation acid was not recorded to be produced from lactose by 5 among 44 cultures.

Milk agar plate: Casein was hydrolyzed with two exceptions.

Potato: The growth was moderate to good, and the color creamy yellow, brown or gray.

Nitrites from Nitrates: Variable, 21 cultures reduced nitrates strongly, no reduction was observed in 23 cultures.

Starch: By Kellerman and McBeth's method[24] hydrolysis of starch could be observed in 22 cultures. From starch in the inorganic base, acid was formed by 42 cultures.

Growth in 5 per cent NaCl broth: All cultures were positive after 3 to 5 days.

Glucose asparagine agar: Asparagine could not be utilized on this media.

Citrate agar: Negative, though in 9 cultures a slightly basic reaction of this medium was recorded.

Glucose broth: Acetylmethylcarbinol was usually not produced. Only 5 among 44 cultures recorded a positive Voges-Proskauer reaction. The final pH after 7 days-incubation was 5.2 to 6.0.

Indole: All cultures were positive.

Hemolysis on blood agar: All cultures possessed marked hemolytic activities.

Löffler's serum: The coagulated serum was liquiefied vigorously by all cul-

tures but one in which the liquefaction was recorded as weak.

Fermentation of carbohydrates: In all cultures acid without gas was recorded from glucose, maltose, dextrin, glycerol, mannose and raffinose. Galactose and sucrose were fermented with each one exception, and also fructose with two exceptions. Acids was formed from lactose by 39 cultures. From salicine acid was produced by 12 cultures. Arabinose, xylose, mannitol, rhamnose and inulin were not fermented.

Discussion

Thirty organisms among 135 of intestinal mesophilic bacteria which formed distinctly bulged sporangia were capable of decomposing thiamin by the supernate of their broth cultures. They and 4 strains of "*Bacillus thiaminolyticus*" sent from Dr. Matsukawa and 10 from Prof. R. Kimura showed similar or quite resembling morphological and cultural characteristics each other, and therefore those thiamin-decomposing bacteria may be grouped in one species. Moreover, we could not succeed to isolate organisms which on account of their cultural and morphological characteristics should belong to this group without having any thiamin decomposing activity. We did not detect any of this activity in the broth cultures of morphologically resembling organisms, and so we could not find any reason to oppose the opinion that these thiamin decomposing bacteria belong to one new species of Genus *Bacillus*, "*Bacillus thiaminolyticus*." *B. thiaminolyticus*, though resembling *B. circulans*, *B. alvei*, *B. laterosporus* and *B. brevis*, differs from the latter in many respects: (1) Situation of the spore in the sporangium is terminal to subterminal, never central. In this respect, *B. thiaminolyticus* relates to *B. circulans* described in Bergey's Manual, 6th Edition. (2) The arrangement of cells and spores of *B. thiaminolyticus* resembles those of *B. alvei*. (3) Amoeboid colonies of *B. thiaminolyticus* like those of *B. circulans* and *B. alvei* have never been recorded. (4) Many cultures of *B. thiaminolyticus* formed thick white pellicle in nutrient broth. Pellicle formation by *B. circulans*, *B. alvei* and *B. laterosporus* was observed with rare exceptions. (5) Indole was produced by all cultures of *B. thiaminolyticus* and by none of organisms belonging to other species, though even in *B. circulans*, *B. alvei* or *B. laterosporus* indole formation could be observed frequently if peptone water had been enriched with 50 mg. tryptophan per 1,000 ml. (6) About the half of the strains of *B. thiaminolyticus* reduced nitrate, while all strains of *B. alvei* were negative. Nitrate were reduced by *B. circulans* and *B. laterosporus* each with rare exceptions. In the Bergey's Manual nitrate reduction is described for *B. alvei* as negative, for *B. circulans* and *B. brevis* as usually positive, and for *B. laterosporus* as positive. (7) Acetylmethylcarbinol was usually not produced by *B. thiaminolyticus*, while many strains of *B. alvei* and none of *B. circulans* produced this substance in glucose broth. According to Bergey's Manual, this substance is formed only by *B. alvei*

among the resembling four species. (8) The ability of *B. thiaminolyticus* to reduce the pH value of glucose broth is in contrast with *B. brevis*, as in the medium acid formed by *B. brevis* is masked by the alkalinity simultaneously produced. (9) All cultures but one of *B. thiaminolyticus* liquefied Löffler's serum vigorously, while no liquefaction was observed among the cultures of *B. circulans*. Organisms belonging to the other three species liquefied this medium frequently. (10) The kinds of carbohydrates decomposed by *B. thiaminolyticus* were quite constant. The fermentation-pattern of *B. thiaminolyticus* resembles that of *B. circulans* and *B alvei*, but there are still some differences between the former and the latter groups. Xylose and arabinose, which are usually fermented by *B. circulans*, and mannitol, on which the reaction of *B. circulans* is usually positive and that of *B. alvei* is variable, are not fermented by *B. thiaminolyticus*. *B. thiaminolyticus* usually produced acid from lactose while all eight cultures of *B. alvei* studied did not ferment this sugar. In Bergey's Manual the reaction of *B. alvei* on this sugar is described as variable.

More recently Kazenlson and Lochhead[25] reported that *B. alvei* required thiamin and could grow in a synthetic medium composed of this vitamin, amino acids, glucose and inorganic salts, while *B. para-alvei* grew in the absence of thiamin yet was stimulated by it. Nakayama[26] found that two strains of *B. alvei* could grow in a synthetic medium containing amino acids, glucose, and inorganic salts without added thiamin, but this vitamin apparently stimulated their growth. He also found that four strains of *B. thiaminolyticus* could grow in the synthetic medium without thiamin and that this vitamin did not promote the growth of them. It may be necessary to study the thiamin-decomposing-activity of *B. para-alvei*, though Smith *et al* [27] considers the latter identical with *B. alvei*.

Summary

From feces of 500 healthy human subjects 135 cultures of mesopilic aerobic bacteria with bulged sporangia were isolated and classified according to the description of Bergey's Manual of Determinative Becteriology, sixth edition, 1948.

There were thirty cultures of *B. thiaminolytcus*, and they had thiamin decomposing activity. In the other cultures no thiaminase activity was detected.

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REFERENCES

(1–11, 13, 20–22 in Japanese)

1) CHANG, C. : Studise on thiaminase in human feces. (1). *Vitamin*, **2** : 174–179, 1949.
2) MATSUKAWA, D. and MISAWA, H. : *Proc. Committ. Res. Vit. B*, 31 : 16, 1949; *Vitamin*, **2** : 265, 1949.
3) MISAWA, H. : Studies on thiaminaase in human feces (X). Finding of a new bacillus which produces thiaminase. *Niigata Med. J.* **65** : 817–820, 1951 : (XI). Bacteriological study on *Bacillus thiaminolyticus*, ditto **66** : 15–18, 1952 : (XII). Thiaminase of Bacteria. ditto, **66** : 93–95, 1952.
4) FUJITA, A., SAIKI, H. and KISHIDA, T. : *Proc. Committ. Res. Vit. B.*, **35** : 6, 1949.
5) FUJITA, A., SAIKI, H., YAMADORI, M. and KISHIDA, T : ditto, **36** : 11, 1950.
6) SAIKI, H., KISHIDA, T., TASHIRO, T. and YAMADORI, M., : Studies on thiaminase (XIV) Bacteriological properties of the actual thiaminase bacilli. *Kitasato Arch. Exp. Med.* **23** : 121–129, 1951.
7) KIMURA, R. and HAYASHI, R. *Proc. Comm. Res. Vit. B.*, **38** : 17, 1950.
8) HAYASHI, R. and ASAKURA, Z. Isolation of aerobic, thiamine destroying and sporeforming bacteria. *Jap. J. Bact.*, **6** : 75–76, 1951.
9) HAYASHI, R. : Studies on the so-called "*Aneurinase Bacillus.* "ditto **6** : 77–79, 1951.
10) KIMURA, R. and HAYASHI, R. : *Proc. Committ. Vit. B.* **43** : 5, 1950.
11) KIMURA, R. and AOYAMA, S. : ditto, **45** : 8, 1951.
12) KUNO, Y. : *Bacillus thiaminolyticus*, a new thiamin-decomposing bacterium. *Proc. Japan Acad.* **27** : 1951, 362–365, 1951.
13) KIMURA R. and AOYAMA, S. : *Proc. Committ. Res. Vit. B.* **47** : 9, 1951.
14) KUNO, Y. : Further studies on thiamin decomposing bacteria. *Proc. Japan Acad.*, **28** : 235–240, 1952.
15) SMITH, . N. R. : Bergey's Manual of Determinative Bacteriology, 6th ed. : 704–762, 1948. Williams & Wilkins Comp. Baltimore.
16) GORDON, R. E. and SMITH, N. R. : Aerobic sporeforming bacteria capable of growth at high temperature. *J. Bact.*, **58** : 327–341, 1948.
17) AYERS, S. H. and JOHNSON, W. T. : A medium for stock cultures of streptoccocci and other bacteria. *J. Bact.*, **9** : 111–114, 1924.
18) SMITH, D. T., MARTIN, D. S., CONANT, N. F., BEARD, J. W., TAYLOR, G., KOHN, H. I. and POSTON, M. A. : Zinsser's Text Book of Bacteriology, 9th ed : 929. Appleton-Century-Crofts, Inc., New York. 1948.
19) WILSON, G. S. and MILES, A. A. : Topley and Wilson's Principles of Bacteriology and Immunity, 3rd. ed : 368–369. Edward Arnold & Co., London., 1946.
20) FUJITA, A., UENISHI, K., TASHIRO, T., YAMASAKI, K., SUGANUMA, J. and KISHIDA, T. : Studies on thiaminase (IX). On the so-called bacterial thiaminase. *Kitazato Arch. Exp. Med.*, **22** : 127, 227, 1949.
21) FUJITA, A., KAMINISHI, K., TASHIRO, T. and YAMASAKI, K. : *Proc. Committ. Res. Vit. B.*, **27** : 6, 1948.
22) NISHIO, M., FUJIWARA, M. and KITAMURA, M. : *Vitamin* **1** : 232–236, 288–292, 1948.
23) GRADWOHOL, R. B. H. : *Clinical Laboratory Methods and Diagnosis*, 4th ed. : 1246–1253.
24) KELLERMAN, K. F. and MCBETH, I. G. : The fermentation of cellulose. *Zbl. Bakt. Parasitenk.*, II, 34, 485–494. Cited by Gordon and Smith (16).
25) KATZENLSON, H. and LOCHHEAD, A. G. : Nutritional requirements of *Bacillus alvei* and *Bacillus para-alvei*. *J. Bact.*, **53** : 83–88, 1947.
26) NAKAYAMA, H. : Unpublished.
27) SMITH, N. R., GORDON, R. E. and CLARK, F. E. : Aerobic mesophilic sporefoming bacteria. *U. S. Dept. Agr., Misc. Pud.* 559, 1946. Cited by Katzenlson and Lochhead (24).