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Requirement of Thiamine for Growth in a Mutant of *Bacillus subtilis*

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Mutants of *Bacillus subtilis* with a heat-sensitive spores have been isolated. Of these mutants, a strain 1508C required simultaneously thiamine and grew normally when thiamine was provided. The mutant have a double requirement for the pyrimidine and thiazole moieties of the vitamin molecule. All the spontaneous revertants isolated from the mutant restores the thiamine deficiency, but does not result in increased heat-resistance of spores, compared with those of wild type.

Introduction

A heat-sensitive sporation mutant was isolated from *Bacillus subtilis* by mutagen treatment. During our investigations on several characters of the mutant, it was found that the mutant had a surprising phenotype, namely a requirement for thiamine. The enzymatic formation of thiamine from 4-amino-5-hydroxymethyl-pyrimidine (OMP) and 4-methyl-5-β-hydroxyethylthiazole (Th) in various microorganisms has been studied in considerable detail (Camiener and Brown, 1960a; Camiener and Brown, 1960b; Nose et al., 1961; Nose et al., 1964; Nakayama and Hayashi, 1971). However, the *de novo* pathways leading to the pyrimidine and thiazole moieties of the thiamine molecule in bacteria remains incompletely known. Newell and Tucker (1968a) reported that by using amino acid-requiring mutants of *Salmonella typhimurium* LT12 pyrimidine moiety of thiamine were synthesized *in vivo* in the presence of both methionine and glycine. Nakayama and Kuba (1970) indicated that requirement for thiamine could be replaced by succinate in a mutant strain of *Escherichia coli* 70–23, which requires thiamine. Newell and Tucker (1968b) found that the thiamine requirement of an Ath^- mutant requiring both adenine and thiamine of *Salmonella typhimurium* was satisfied by the purine precursor, 4-aminoimidazole riboside phosphate. The studies of Watanabe et al. (1981) with mutant of *Escherichia coli* D–30 requiring the pyrimidine moiety of thiamine revealed that the requirement for the pyrimidine moiety could be replaced by methionine and lysine, but not by succinate. Walter and Backer (1977) reported that from the culture fluid of a mutant growing on the thiazole moiety, two compounds were isolated and identified as OMP and its monophosphate derivative

in a thiamine-deficient mutant of *Bacillus subtilis*. In this paper, we describe characters of thiamine-requiring mutant of *Bacillus subtilis*.

Materials and Methods

Bacterial strains

Bacillus subtilis RIMD and its mutant 1508C were used throughout this experiments. The mutants were isolated after treatment of the parental strain with N-methyl-N-nitro-N-nitrosoguanidine. The mutagene-treated cultures was plated on nutrient agar medium and colonies with a different pigmentation from the normal brown were picked up and a heat-resistance of spores obtained from each colony were examined for 30 min at 80°C. Mutant 1508C with a heat-sensitive spores was selected from colonies. The mutant was simultaneously characterized as thiamine requiring (describe in Results).

Growth condition

The wild and mutant strains of *Bacillus subtilis* were maintained on nutrient agar medium. Both strains were cultivated at 37°C in nutrient broth medium (NB medium) consisting of 1% peptone, 1% Erlich meat extract and 0.5% NaCl (pH 7.0). For the determination of the growth response in various media, each the cells was harvested by centrifugation at 8,000 rpm for 10 min, and then washed and resuspended in peptone medium consisting of 1% peptone, 5 mM $CaCl_2$ and 0.4% glucose (pH 7.0) and inoculated in the suitable media. The growth responces in the various media were monitored by absorbance at 600 nm.

Preparation of OMP

4-amino-5-hydroxymethyl-2-methylpyrimidine (OMP) were preparated from thiamine hydrochloride by the method of Watanabe (1936) with minor modification. Thiamine hydrochloride solution was hydrolyzed by autoclaving at 125°C for 6 hrs. and the hydrolysate was separated by thin-layer chromatography by the method of Neal (1969). Separated spots were collected and extracted with water. Absorption spectra of the extracted samples were measured and the sample which had the same Rf values and absorption maximums as OMP was used in this experiment.

Selection of spontaneous prototrophic revertants of the mutant

The mutant was grown overnight in nutrient broth. The bacteria was washed and resuspended in peptone medium solution, and revertants to thiamine prototrophy were selected on unsupplemented peptone agar medium.

Preparation of spores

The wild, mutant and revertant strains of *Bacillus subtilis* were grown in NB agar medium. The cultures were harvested when release of free spores was usually complete. The harvested spores were suspended in distilled water or NB medium and, if not used directly, were stored as a paste at −10°C.

Chemicals

Heart infusion medium, peptone, Erlich meat extract were obtained from Kyokuto Pharmaceutical Industrial Co., Tokyo. Yeast extract powder was obtained from Oriental Yeast Industrial Co., Tokyo. 4-methyl-5-hydroxymethylthiazole, N-methyl-N-nitro-N-nitrosoguanidine were purchased from Sigma Chemical Company. All vitamins used in this experiment except for 4-methyl-5-hydroxymethylthiazole were obtained from Kanto Chemical Co., Ltd. All nucleic acids were obtained from Nakarai Chemicals Ltd.

Results

Comparative physiological characteristics of wild and mutant strains

The mutant has a high degree of similarity to wild type in its physiological characters (Table 1). On the other hand, the mutant did not grow at 45°C, but the wild type grew adequately under the same condition.

Table 1. Comparative physiological characteristics of *Bacillus sub.* mutant and wild strains

Physiological test	Mutant	Wild
V–P reaction	+	+
Catalase	+	+
Growth in 7% NaCl	−	+
Growth at pH 6.0	+	+
Growth at 55°C	−	−
Growth at 45°C	−	+
Casein hydrolysis	+	+
Starch hydrolysis	+	+
Gelatin hydrolysis	+	+
Nitrite from nitrate	+	+

Symbols: +, growth or positive test; −, no growth or negative reaction.

Table 2. Effect of the culture medium on growth of the mutant and wild strains

Medium	Growth (A_{600})	
	Mutant	Wild
Spizizen	0.05	0.88
Peptone broth	0.11	1.25
Nutrient broth	1.90	2.60
BHI	2.50	2.80

The mutant and wild strains were incubated to four different media at initial cell densities of 0.07 (A_{600}), respectively. At 8 hr after inoculation, the A_{600} was measured.

Growth

The growth responses of the mutant and wild strains in various media were investigated (Table 2). The mutant strain grew normally in BHI and NB medium, but not in Spizizen or peptone medium, compared with wild type. Further, the mutant strain could not grow in Spizizen medium even if succinate or lysine and methionine were supplied at concentration of 0.1%, respectively. The growth rates of the mutant were compared in the P medium with or without yeast extract powder (0.5%) (Fig. 1a). It was found that the mutant strain did not grow in the P medium, unless yeast extract was added to the medium. Yeast extract powder contains vitamins and nucleic acid materials as a component.

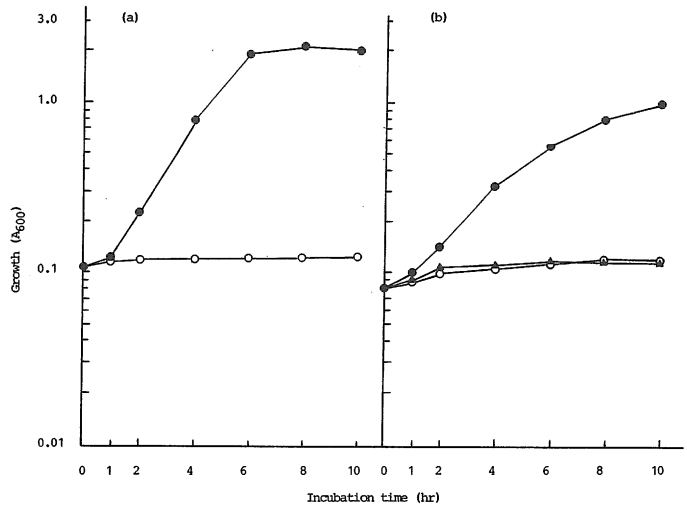

Fig. 1. Growth responses to the mutant to yeast extract (a), and vitamines and nucleic acid materials (b). The mutant was grown in P medium supplemented as indicated; (a), ○——○: No additions; ●——● :with yeast extract 0.4%, and (b), ○——○: no additions; ●——●: vitamins bases (biotin, folic acid, inosine, nicotinic acid, pantothenic acid, pyridoxin, riboflavin and thiamine, 15 μg/ml, respectively); ▲——▲; nucleoside and nucleobases (adenosine, uridine, cytosine, guanine, hypoxanthine, thymine and xanthine, 15 μg/ml, respectively).

Growth of the mutant in the P medium with added vitamins bases (biotin, folic acid, inosine, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine) or nucleoside and nucleobases (adenosine, uridine, cytosine, guanine, hypoxanthine, thymine and xanthine) was studied. Figure 1b showed that the growth was stimulated only by addition of vitamins base, except for nucleoside, purine and pyrimidine bases. The effects of various vitamins base for the growth were examined in the P medium.

The mutant grew only when thiamine was present, whereas none of the other substrate tested had any effect (Table 3).

It is known that the pyrimidine and thiazole moieties of thiamine molecule are synthesized *via* independent metabolic pathway. Effects of the pyrimidine and thiazole for the growth were tested, alone or in combination. Figure 2 indicated that the mutant required the simultaneous presence of the pyrimidine and thiazole for growth. These results show that the gene(s) of this thiamine-deficient mutant was blocked in certain steps up to the biosynthesis of 4-methylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine, respectivery.

Table 3. Effect of various vitamins on growth of the mutant

Vitamins	Growth (A_{600})	
	6 hr	12 hr
Control	0.14	0.14
Biotin	0.14	0.13
Folic acid	0.14	0.14
Inosine	0.15	0.14
Nicotinic acid	0.15	0.14
Pantothenic acid	0.13	0.12
Pyridoxine	0.14	0.13
Riboflavine	0.13	0.13
Thiamine	0.53	0.84

The mutant and wild strains were cultured in P medium supplemented with indicated vitamin bases (15 μg/ml, respectively). Initial cell density was 0.08 (A_{600}). After 6 and 12 hr of incubation, cell growth was measured.

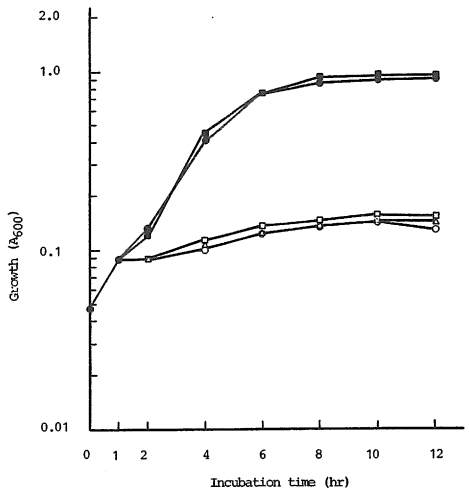

Fig. 2. Pyrimidine and thiazole requirement of the mutant growing in P medium. Symboles, ○——○: No additions; ●——●: with yeast extract 0.4%; □——□: with pyrimidine (OMP), 15 μg/ml △——△: with thiazole (Th), 15 μg/ml; ■——■: with pyrimidine (OMP) and thiazole (Th), 15 μg/ml, respectively.

Isolation of revertant and its properties

The mutant was analysed by reversion tests. Spontaneous prototrophic revertants were obtained from the mutant at a frequency of ca. 10^{-8}. The mutant also spontaneously segregated revertants growing on hydroxymethylpyrimidine in the absence of thiazole and on thiazole in the absence of hydroxymethylpyrimidine at a similar frequency. All the revertants isolated no longer required thiamine. Figure 3 show growth of a revertant strain. These results suggest that the mutant

with a double requirement for the pyrimidine and thiazole moieties may be single mutant.

As one of other characters of revertant strains, heat-susceptibility of revertant type spores was compared with those of the mutant and wild type spores (Fig. 4). Unlike the wild spores, revertant spores was very sensitive to heat as well as the mutant spores.

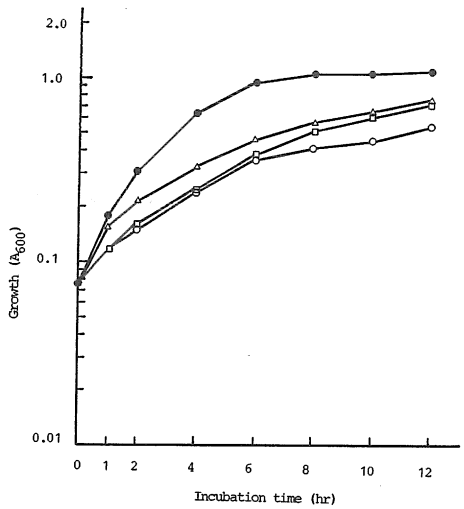

Fig. 3. Growth of revertant strain in P medium. Symbols, ○——○: No additions; □——□: with pyrimidine (OMP), 15 μg/ml; △——△: with thiazole (Th), 15 μg/ml; ●——●: with pyrimidine (OMP) and thiazole (Th), 15 μg/ml, respectively.

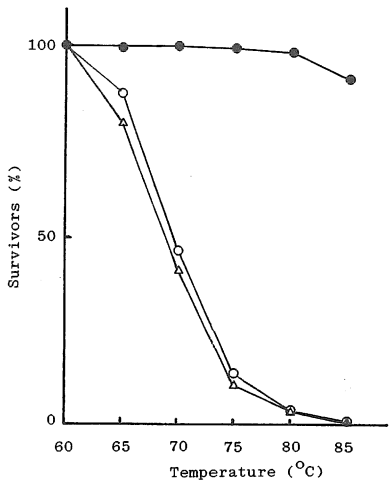

Fig. 4. Effect of each temperature on heat resistance of the revertant, mutant and wild type spores. Harvested spores were suspended in distilled water. Then, the samples were removed and heated at temperature ranging from 60 to 85°C for 15 min. The samples were diluted and plated in triplicate on NB agar medium and counted after incubation overnight at 37°C. Symbols, ○——○: The mutant; △——△: the revertant: ●——●: wild spores.

Discussion

Auxotrophic mutants of microorganisms which require specific growth factor for their growth have been very useful for the elucidation and analysis of biosynthetic pathways. A heat-sensitive sporation mutants isolated in our laboratory were found to be simultaneously deficient in thiamine biosynthesis. Another characters of the

mutants differ markedly from those of wild type: the mutant spores were more sensitive to heat and sodium laurylsulfate (SDS) than wild type, but equally resistant to lysozyme (Unpublished data).

Johnson et al. (1966) indicated that in baker's yeast at least the metyl carbon and the sulfur of methionine were incorporated in the thiazole structure. Recently, a mutant strain of *Escherichia coli*, D–30, requiring the pyrimidine moiety of thiamine for growth in a minimal medium supplemented with the thiazole moiety of thiamine was isolated (Watanabe et al. 1981). They showed that the requirement for the pyrimidine moiety could be replaced by methionine and lysine, but not by succinate. In this paper, we indicated that the requirement for thiamine could not replaced by methionine and lysine or succinate in the mutant of *Bacillus subtilis*. Muller–Falcke (1974) reported that mutant of *Sacch. cerevisiae* with a double requirement for the pyrimidine and thiazole moieties was resulted from mutation in one of several unliknked genes. Walter and Backer (1977) suggested that in *Bacillus subtilis*, mutants with a double requirement for the pyrimidine and thiazole moieties was caused by double mutation in two different thiamine genes. Recently, mutants of *Escherichia coli* K12 deficient in thiamine and L-serine deaminase activity have been isolated, and from a study of the revertants and transductants a single mutation was responsible for the thiamine requirement and for the decrease in L-serine deaminase activity (Newman et al. 1985). In this paper, spontaneous prototrophic revertants were obtained at a similar frequency to revertants growing on the pyrimidine or thiazole. These results suggest that the mutant of *Bacillus subtilis* with a double requirement for the pyrimidine and thiazole moieties was resulted from a single mutation. Furthermore, spontaneous revertants of the mutant restores the thiamine deficiency, but does not result in increased heat-resistance of spores, compared with those of wild type. The exact map position of the mutation remains to be established, but the availability of a large number of such mutants may also allow further study of the thiamine pathway, which is not known.

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