Studies on the Biosynthesis of Vitamin B_6 in *Bacillus subtilis*

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CONTENTS

INTRODUCION

Vitamin B_6 (B_6), a water-soluble vitamin, was first discovered as an anti-dermatitis factor of rats (Gyogy, 1934), and then, six forms of B_6, pyridoxine (PN), pyridoxine 5'-phosphate (PNP), pyridoxal (PL), pyridoxal 5'-phosphate (PLP), pyridoxamine (PM), and pyridoxamine 5'-phosphate (PMP), were defined (Rabinowitz and Snell, 1941) (Fig. 1).

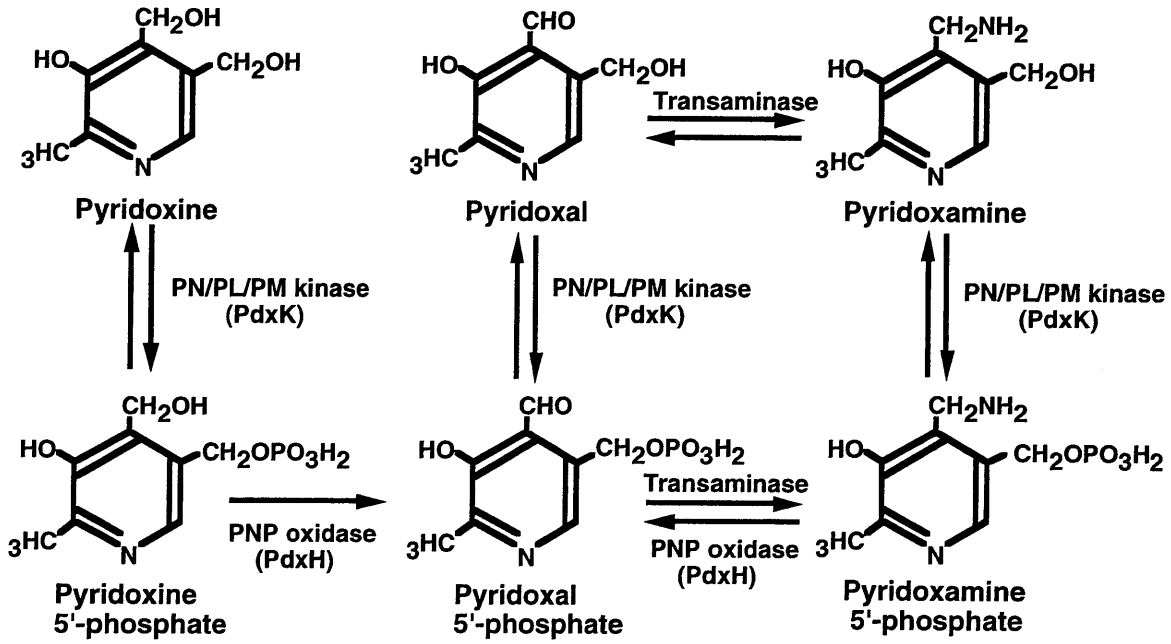

Fig. 1. The salvage pathway for B6

Subsequently, an important role of B_6 was first found (Gunsalus and Bellamy, 1944). Among six forms of B_6, PLP was required for decarboxylation and transamination of amino acids as a coenzyme. Since then, it has been recognized that PLP plays an important role in metabolic reactions of various amino acids and others. To earn the active coenzyme form of B_6, microorganisms and others convert from inactive forms to PLP *via* the salvage pathway. The pathway serves to convert from whatever the forms of B_6 enter the cell to the active coenzyme form, PLP (Fig. 1).

The *de novo* biosynthesis of B_6 has been studied with *Escherichia coli* and also other microorganisms. PN is thought to be the first product among B_6 compounds in the biosynthetic pathway. However, the biosynthetic pathway has been scarcely elucidated in spite of extensive studies. The small amount of PN and its precursors in cells makes difficult elucidation of the biosynthetic pathway.

Elucidation of B_6 biosynthetic pathway was started with genetic, nutritional, and isotopic labeling experiments on *E. coli*. There is an abundant amount of data from genetic and isotopic labeling analysis that limits the possible compounds involved in the biosynthetic pathway. There are also several nutritional analyses that give clues about the pathway.

For the first step to define precursors and genes of B_6 biosynthetic pathway, approximately 250 pyridoxine auxotrophs (*pdx* mutants) of *E. coli* were derived (Dempsey, 1969). Those *pdx* mutants fell into five unlinked groups widely distributed on the chromosome, and characteristics of each group were described. Mutants belong to group I (*pdxB, C, D* mutants) grow with either PN or glycolaldehyde (Dempsey, 1971). The characteristic of group II is unknown, but these mutants are believed to be *pdxA* or *pdxG* mutants. Mutants belong to group III require both serine and PN to grow, and lack 3-phosphoserine-oxoglutarate transaminase. Those mutants are known as p*dxF* or *serC* mutants (Dempsey, 1969; Shimizu and Dempsey, 1978). High level of 3-hydroxypyruvate can replace PN. Mutants belong to group IV required PL or PM, and lacked PNP oxidase. These mutants are known as *pdxH* mutants (Dempsey, 1966, 1971a). Mutants belong to group V fell into two phenotypes. One requires PN that is known as *pdxJ* mutants. The other mutants grow partially with D- or L-alanine that are known as *pdxK* mutants (Dempsey, 1972).

Isotopic labeling experiments were performed on two of *E. coli* B *pdx* mutants. One is a group I mutant (*pdxB*), WG3 strain, that is a simple prydoxineless mutant. The other one is a group IV mutant, WG2 strain, that is deficient in the salvage pathway but wild type for synthesis of PN. Spenser and his co-workers tested the incorporation of glycerol, pyruvate, and L-serine in the WG2 strain (Hill *et al.*, 1971, 1972,1975, 1977). C-1 of glycerol labeled C-2', -3, -4', -5, and -6 atoms of PN whereas C-2 of glycerol labeled C-2, -4, and -5 atoms of PN. It was also established that C-3 of pyruvate labeled C-2' atom of PN. Similarly, L-serine labeled C-2' atom of PN (Vella *et al.*, 1981).

Tani and Dempsey (1973) tested the incorporation of glycolaldehyde in WG3 strain using radioactive glycolaldehyde. C-1 of glycolaldehyde was incorporated into C-5 of PN, and C-2 of glycolaldehyde was incorporated into C-5' of PN without any detectable dilution (Tani and Dempsey, 1973; Hill *et al.*, 1977). It is obvious that the *pdxB* mutation blocks synthesis of the compound that glycolaldehyde somehow replaces.

Biochemical status of GA was also studied. From enzymatic studies, it became apparent that glycolaldehyde is synthesized from glycolate by glycolaldehyde dehydrogenase (GADH) in *E. coli* (Tani *et al.*, 1978). There are three isozyme (A, B, and C) of GADH in the crude extract of *E. coli* B, but only isozyme A in WG3 strain. Synthesis of isozyme B and C were completely repressed by PLP, but isozyme A was not. PN inhibited the activity of isozyme C and activated isozyme B, but did not affect the activity of isozyme A. From these results, it was suggested that isozyme B and C are involved in B_6 biosynthesis, but isozyme A is not (Tani *et al.*, 1978). A similar enzyme, glycolate reductase, was found in the cell-free extract of a potent B_6 producer, *Flavobacterium* sp. 238-7 (Tani *et al.*, 1984). The enzyme seemed to lack the feedback control systems, and it might explain the overproduction of B_6 (Tani *et al.*, 1984).

As a result of above studies, Tani suggested that glycolaldehyde is a precursor of B_6 and proposed a hypothetical biosynthetic pathway for B_6 (Tani, 1977). According to the pathway, glycolaldehyde and a C4 compound form α, β-dihydroxy-β-hydroxymethylvalerate, then a C3 compound is added to form PN. In this pathway, glycolaldehyde can be explained reasonably to enter the 5-5' position of B_6.

Besides above nutritional, isotopic labeling and biochemical studies, Winkler and his co-workers have been working on *E. coli* K-12 using molecular biological techniques, and proposed a biosynthetic pathway (Fig. 2)(Lam and Winkler, 1990). The pathway consists of two branches. On one branch, 1-deoxy-D-xylulose is synthesized from pyruvate and glyceraldehyde by Dxs (1-deoxy-D-xylulose synthase). The other branch starts with erythrose 4-phosphate and leads to the intermediate 4-hydroxy-L-threonine. Formation of 4-hydroxy-L-threonine is catalyzed by TktA, B, GapA,B, PdxB and SerC. (TktA, B are transketolase, GapA, B are glyceraldehyde 3-phosphate dehydrogenase, PdxB is 4-phospho-erythronate dehydrogenase and SerC is phosphoserine aminotransferase.) After decarboxylation of 4-hydroxy-L-threonine, two intermediates are joined into PN by PdxA and PdxJ. Spencer showed that the C5 unit C-2', -2, -3, -4, -4' and the C3N unit N-1, C-6, -5, -5' of pyridoxine are derived from 1-deoxy-D-xylulose and 4-hydroxy-L-threonine, respectively in *E. coli* B WG2 strain (Kennedy *et al.*, 1995).

The roles of PdxA and PdxJ in the proposed pathway were also studied. PdxA catalyzes the NAD^+-dependent oxidation of 4-(phosphohydroxy)-L-threonine to 3-phosphohydroxy-1-aminoacetone (Cane *et al.*, 1998). PdxJ catalyzes the condensation of 3-phosphohydroxy-1-aminoacetone and 1-deoxy-D-xylulose 5-phosphate to yield PNP (Cane *et al.*, 1999). Incubation of PdxJ with PdxA, 4-(phosphohydroxy)-L-threonine,

NAD^+ and 1-deoxy-D-xylulose 5-phosphate resulted in the formation of PNP (Laber *et al.*, 1999).

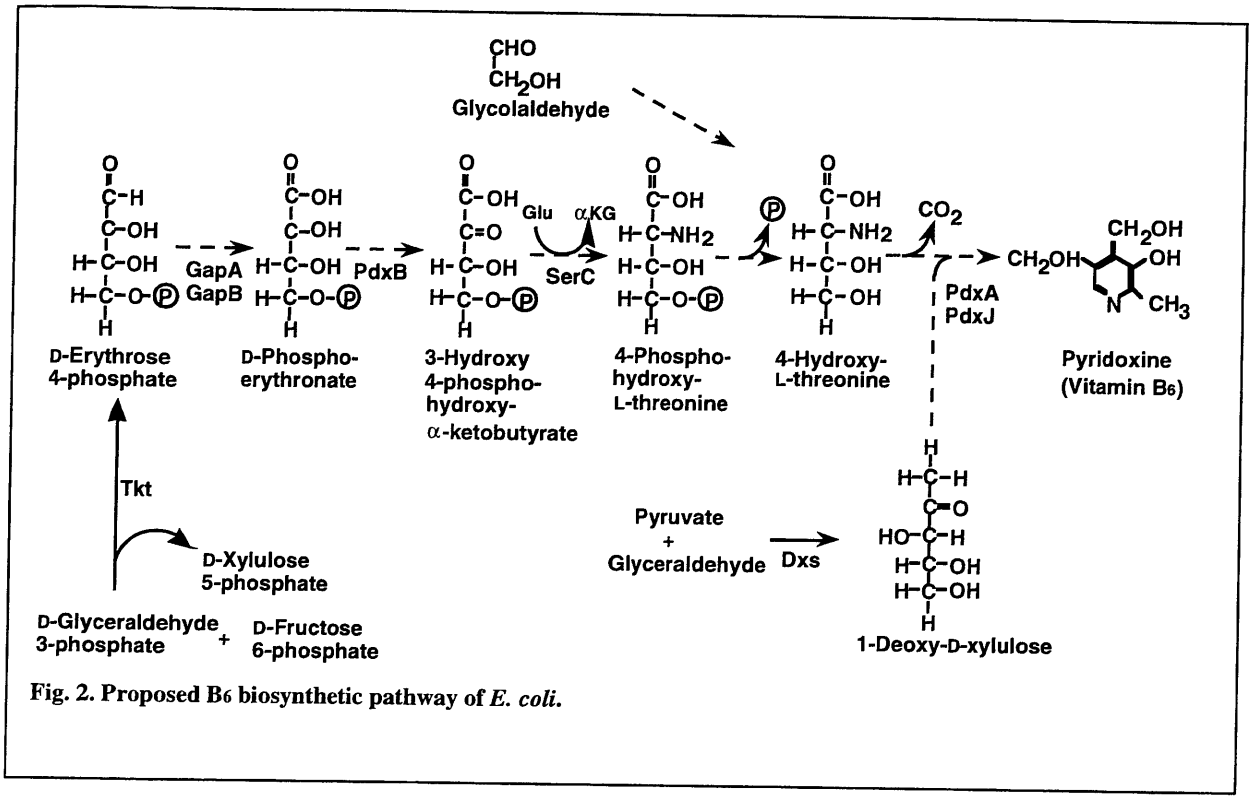

Fig. 2. Proposed B6 biosynthetic pathway of *E. coli*.

However, above proposed pathway still remains unclear. No tracer experiment was performed on the intermediates in the proposed pathway of *E. coli*. For example, erythrose 4-phosphate is considered to be the starting compound in the pathway, but there is no evidence that erythrose 4-phosphate is incorporated into the pyridoxine ring. The same things can be said to other proposed intermediates. In addition, no direct evidence to prove the involvement of the genes in the proposed pathway was shown. SerC is considered to be involved in the pathway, but no enzymatic study was performed. There is no evidence that SerC catalyzes the reaction in the pathway. Dxs is known to

synthesize 1-deoxy-D-xylulose, but there is no evidence that the enzyme is involved in B_6 biosynthesis.

In addition to above studies on *E. coli*, genes involved in B_6 biosynthesis in microorganisms other than *E. coli* were reported. *SOR1* gene of *Cercospora nicotianae* and *pyroA* of *Aspergillus nidulans* were possibly involved in B_6 biosynthesis. *SOR1* gene was identified as a gene involved in resistance of *C. nicotianae* to singlet-oxygen-generating phototoxins, and the growth of *sor1* mutant of *C. nicotianae* was supported by pyridoxine (PN) (Ehrenshaft *et al.*, 1999). Similar result was obtained in *A. nidulans* (Osmani *et al.*, 1999). Mutation of *pyroA*, the homologue of *C. nicotianae SOR1*, caused increased sensitivity to singlet oxygen generated by photosensitizer, and addition of PN reversed the toxicity. Both *SOR1* and *pyroA* are considered to be the member of highly conserved *SNZ* family. *SNZ* genes were originally identified as genes expressed during the stationary phase in *Saccharomyces cerevisiae*, each of which has another highly conserved gene, *SNO*, upstream (Fuge *et al.*, 1994; Braun *et al.*, 1996; Padilla *et al.*, 1998).

It is obvious that *SOR1* and *pyroA* mutants require B_6. However, B_6 amounts synthesized by these mutants were never measured. The functions of *SOR1* and *pyroA* are still unknown, and even the function of the *SNZ* family is unknown. The *SNZ* mutant of *S. cerevisiae* does not require B_6, and therefore, the function of SOR1 and PyroA could be different from that of *SNZ* of *S. cerevisiae*. Together, the involvement and the role of *SOR1* and *pyroA* in B_6 biosynthesis are still uncertain.

Biosynthetic pathway of B_6 in *Rhizobium meliioti* was also proposed based on labeling experiments (Tazoe *et al.*, 2000). The pathway is similar to the pathway of *E. coli* proposed by Winkler. The proposed precursors of B_6 in *R. meliioti* are 1-deoxy-D-

xylulose and 4-hydroxy-L-threonine. Like *E. coli*, 1-deoxy-D-xylulose is formed from glyceraldehyde and pyruvate. On the other hand, unlike *E. coli*, 4-hydroxy-L-threonine is formed from glycine and glycolaldehyde. However, no enzymatic studies were shown to prove the proposed pathway yet.

Besides the searches for precursors, origins of the nitrogen atom of B_6 were studied in various microorganisms (Tanaka *et al.*, 2000). The amide nitrogen atom of glutamine was incorporated into PN in eukaryotes, *Emericell nidulans*, *Mucor racemosus*, *Neurospora crassa* and *S. cerevisiae*. Nitrogen atom of glutamine was also incorporated in prokaryotes, *Staphylococcus aureus* and *B. subtilis*. On the other hand, the nitrogen atom of gluamate was incorporated into PN in prokaryotes, *Pseudomonas putida*, *Entrobacter aerogenes* and *E. coli*. These microorganisms were divided into two groups, depend on origins of nitrogen atom, but not depend on prokaryotes and eukaryotes. These results suggested that there could be different biosynthetic pathways of B_6 among microorganisms.

In this thesis, the biosynthesis of B_6 in *Bacillus subtils*, was studied. As compared to *E. coli*, little study of B_6 biosynthetic pathway of a gram-positive bacterium, *B. subtilis*, was done. There are only few reports on B_6 biosynthetic pathway of *B. subtilis*, and no reliable information was reported. The sequence of whole *B. subtilis* chromosome was determined (Kunst *et al.*, 1997). The chromosome is 4,214,814 bp long, and encodes 4107 protein genes. However, no gene was found to be involved in B_6 biosynthesis.

Elucidation of biosynthetic pathways usually starts with construction and collection of various auxotrophic mutants followed by nutritional and isotopic labeling experiments. Therefore, the isolation of B_6 auxotrophs of *B. subtilis* was fast attempted using with various mutagenesis methods such as ultra violet, nitrosoguanidine, ethyl

methanesulfonate and transposon mutagenesis. Approximately 20,000-100,000 colonies were assayed for each method, and approximately 1% of them showed auxotrophy. However, no B_6 auxotroph was derived. The reason is unknown, but one of the reasons is assumed to be that the amount of B_6 required by *B. subtilis* is very low, and therefore, it was very difficult to distinguish B_6 auxotrophs from the wild-type strain. The poor growth of *B. subtilis* on minimal medium agar plates also made the isolation of B_6 auxotrophs difficult.

Regarding isotopic labeling experiments, in author's knowledge, labeling experiments on the wild-type strains of *E. coli* were never successful. Even on B_6 auxotrophs, the labeling experiments have not led to the unequivocal identification of precursors, except for GA. Therefore, it was unlikely that labeling experiments on the wild-type strain of *B. subtilis* would lead to the identification of precursors.

Therefore, elucidation of B_6 biosynthetic pathway of *B. subtilis* was primary attempted using information from other microorganisms. Recent progress of genome analysis showed the full sequence of *B. subtilis* chromosome in 1997 (Kunst *et al.*), and DNA manipulating techniques of *B. subtilis* were rapidly developed. However, when this work was started, the determination of the full sequence of *B. subtilis* chromosome was not completed, and no homologue of *pdx* genes or *serC* was found in the published genome sequence.

Because of the lack of information of genes, this work was started from investigation of the only confirmed precursor, GA. Without auxotrophic mutants and the complete gemone sequence of *B. subtilis*, investigation of the GA-forming route was the only way to start investigating the B_6 biosynthetic pathway. As the research on the GA-forming

route progressed, the full sequence of *B. subtilis* chromosome was completed. It became apparent that there are homologues of *serC* and tkt, but *pdx* genes.

Without B_6 auxotrophs, there was no way to identify the precursors of B_6. The only way to study the pathway was to identify the genes involved in B_6 biosynthesis by the investigation of the genes, which might be involved in B_6 biosynthesis of other microorganisms.

The isolations of B_6 auxotrophs of *B. subtilis* were always attempted parallel to other works. The isolation of B_6 auxotrophs of the *serC* disruptant was also attempted because there could be two pathways, the proposed pathway of *E. coli* and an unknown pathway. In the *serC* disruptant, the former pathway is blocked, and therefore, genes involved in the unknown pathway could be isolated. However, B_6 auxotrophs of *B. subtilis* were never derived despite of extensive searches.

Based on above research background, in this thesis, the author primary focused on investigations of the GA-forming route and genes involved in B_6 biosynthesis of other microorganisms.

In Chapter I, the GA-forming route, in which GADH or another GA-forming enzyme is possibly involved, was examined. In Chapter II, involvement of *serC* was examined. *serC* was disrupted in *E. coli* and *B. subtilis*, and characteristics of two strains were compared. In Chapter III, general characteristics of the *tkt* disruptant to know the involvement of *tkt* were also studied. In Chapter IV, conditional mutants of *dxs* and *dxr* were derived to examine the involvement of *dxs* and *dxr*. In Chapter V, involvement of *yaaD* and *yaaE* was demonstrated.

CHAPTER I

Glycolaldehyde-Forming Route in *Bacillus subtilis* in Relation to Vitamin B_6 Biosynthesis

INTRODUCTION

Numerous genetic, nutritional and isotopic labeling experiments have been performed on vitamin B_6 (B_6) biosynthesis with *Escherichia coli.* According to genetic experiments, B_6 auxotrophs (*pdx* mutants) of *E. coli* fell into five unlinked groups (I-V) widely distributed on the chromosome. It was found that B_6 can be substituted by glycolaldehyde (GA) on *E. coli* B WG3 strain (Dempsey, 1971), which belongs to group I of *pdx* mutants. It was shown that the C-1 atom of the GA molecule was incorporated into C-5 of pyridoxal 5'-phosphate, and C-2 into C-5' without any detectable dilution (Fig. 1.1)(Hill *et al.*, 1975; Tani and Dempsey, 1973).

In *E. coli* B, GA is synthesized through the reduction of glycolate by GA dehydrogenase (GADH)(Fig. 1.1)(Tani *et al,* 1978). It was also found that GADH activity is widely distributed in bacteria. Glycolate reductase activity was detected in the cell-free extract of a potent B_6 producer, *Flavobacterium* sp. 238-7 (Tani *et al,* 1984). The glycolate reductase was not controlled by B_6 with feedback inhibitions or repression, explaining the overproduction of B_6 (Tani *et al,* 1984).

Until now, little has been studied regarding the B_6 biosynthesis of *Bacillus subtilis.* In this chapter, GA-forming route in relation to vitamin B_6 biosynthesis in *B. subtilis* was investigated.

phosphate buffer (KPB), pH 7.0 and the enzyme in a total volume of 3.0 ml. The reduction of glycolate by GADH was assayed at 37℃ for 5 min in the reaction mixture containing 75 μmol of Na·glycolate, 0.9 μmol of NADPH, 300 μmol of KPB, and the enzyme in a total volume of 3.0 ml. To assay for NADH/NADPH oxidase activity, Na·glycolate was removed from the above reaction mixture. Protein was determined according to the method of Bradford (Bradford, 1976) with a protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as the standard. One unit of the enzyme activity was defined as the amount of the enzyme which oxidized/reduced 1 nmol of NADPH/$NADP^+$ per min in the assay system. The specific activity was expressed as units per mg of protein.

Crude extract preparation

B. subtilis was cultivated in a medium containing 1.0% (w/v) soluble starch, 1.3% NH_4NO_3, 0.2% yeast extract, 0.5% K_2HPO_4, 0.1% KH_2PO_4, 0.2% NaCl and 0.02% $MgSO_4 \cdot 7H_2O$, pH 7.0. The cultivation was carried out with a desktop jar fermentor system (Mitsuwa Biosystem Co., Osaka) at 30℃, 400 rpm, and 1.0 volume per volume per min. The cells were harvested during the early stationary phase and disrupted with 0.25-mm glass beads using Bead-Beater (Biospec Products, Bartlesville, OK). Cell debris was removed by centrifugation (100,000 × *g* for 30 min at 4℃), and the resulting supernatant was designated as the crude extract.

Purification of enzymes

GADH was purified using the following procedure: the crude extract was applied to a DEAE-Sephacel (Amersham Pharmacia Biotech UK Ltd., England) column, and eluted by a linear gradient of NaCl from 0.1 to 1.0 M in KPB. Active fractions were collected

and brought to 50% saturation with solid $(NH_4)_2SO_4$. Precipitates formed were removed by centrifugation and applied to a Butyl-Toyopearl 650S (Tosoh Corp., Tokyo) column, and eluted by a linear gradient of $(NH_4)_2SO_4$ from 50% to 0% saturation in KPB. Active fractions were collected.

RESULTS

Purification of GADH from *B. subtilis* IFO 3007

To investigate the reducing activity of GADH, GADH was purified as described in MATERIALS and METHODS. Throughout the procedures, the apparent oxidizing activity of GADH was measured. The second active fraction from DEAE-Sephacel column chromatography (Fig. 1.2) was further purified by $(NH_4)_2SO_4$ fractionation and Butyl-Toyopearl column chromatography. The purification procedure is summarized in Table 1.1. GADH was purified 52.0-fold with a yield of 81.8% by the procedure. However, its GA-forming activity could not be measured because of the coexisting high NADH/NADPH oxidase activity. Therefore, it was necessary to eliminate NADH/NADPH oxidase for the investigation of the reducing activity of GADH.

TABLE 1.1. Purification of GADH from *B. subtilis* IFO 3007

Fractionation step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	1070	192	0.179	1	100
DEAE-Sephacel	324	691	2.16	12.1	360
$(NH_4)_2SO_4$	172	396	2.30	12.8	206
Butyl-Toyopearl	16.8	157	9.31	52.0	81.8

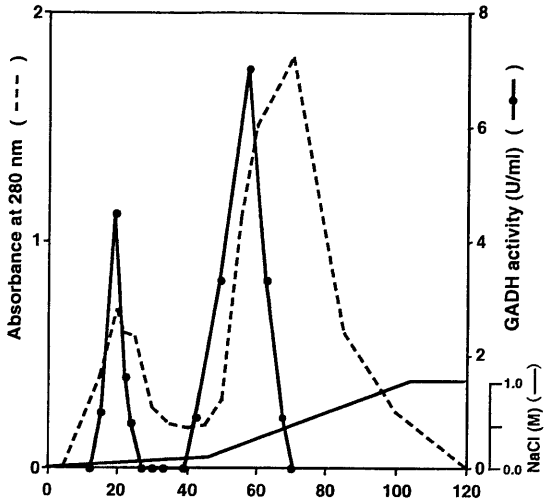

FIG. 1.2. DEAE-Sephacel column chromatography of GADH from *B. subtilis* IFO 3007.

SECTION 2. Purification of NADH/NADPH oxidase

In section 1, it was shown that NADH/NADPH oxidase had to be eliminated to investigate the reducing activity of GADH (GA-forming activity). Therefore, in this section, NADH/NADPH oxidase was purified, and the gene encoding NADH/NADPH oxidase was determined. The enzymatic characteristic of NADH/NADPH oxidase was also investigated.

MATERIALS AND METHODS

Purification of NADH/NADPH oxidase

NADH/NADPH oxidase was purified using the following procedure: the crude extract was applied to a DEAE-Sephacel (Amersham Pharmacia) column, and eluted by a linear gradient of NaCl from 0.1 to 1.0 M in KPB. Active fractions were collected and brought to 30% saturation with solid $(NH_4)_2SO_4$. Precipitates formed were removed by centrifugation and applied to a Butyl-Toyopearl 650S (Tosoh) column, and eluted with a linear gradient of saturated $(NH_4)_2SO_4$ from 30% to 0% in KPB. The buffer concentration of pooled active fractions was diluted to 10 mM, and the pH was adjusted to 6.8. Then, the enzyme solution was applied to a Gigapite (Seikagaku Kogyo Co., Tokyo) column, and eluted with a linear gradient of KPB (pH 6.8) from 10 mM to 500 mM. Active fractions were applied to a Toyopearl HW-65 (Tosoh) column and eluted with KPB containing 0.25 M NaCl. Pooled active fractions were applied to a Mono Q HR 5/5 (Amersham Pharmacia) column and eluted with a linear gradient of NaCl from 0 to 0.6 M in KPB. Active fractions were again applied to a Mono Q HR 5/5 column and

eluted with a linear gradient of NaCl from 0 to 0.4 M in KPB. Active fractions were collected.

Electrophoresis

Polyacrylamide gel electrophoresis (native PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS- PAGE) were carried out with a Phastsystem (Amersham Pharmacia). Protein samples were run on PhastGel Homogeneous 20 (native PAGE) and PhastGel gradient 10-15 (SDS-PAGE), and stained with PhastGel BlueR (Amersham Pharmacia). A molecular weight marker kit (Oriental Yeast Co., Tokyo) was used as the molecular weight standards for SDS-PAGE.

Molecular mass determination

The molecular mass of the native enzyme was estimated by gel permeation column chromatography with Superose 12 HR 10/30 (Amersham Pharmacia). The standard proteins used were ferritin (450 kDa), rabbit muscle aldolase (158 kDa), bovine serum albumin (68 kDa), hen egg albumin (45 kDa), and bovine pancreas chymotrypsinogen A (25 kDa), from a molecular weight calibration kit, combithek (Boehringer Mannheim Biochemica, Germany)

N-terminal and internal amino-acid sequencing

Purified NADH/NADPH oxidase was transferred to a PVDF membrane using ProSorb (PE-Applied Biosystems, Wellesley, MA), and N-terminal amino acid sequence was determined using a 476A protein sequencer (PE-Applied Biosystems). For the determination of internal amino acid sequence, purified NADH/NADPH oxidase was digested with lysyl endopeptidase (Wako Pure Chemical Industries, Osaka). Digested enzyme was blotted onto a PVDF membrane using a 173A MicroBrotter (PE-Applied

Biosystems). Amino-acid sequence of well separated 4 peptides were determined with a 476A protein sequencer (PE-Applied Biosystems).

RESULTS

Purification of NADH/NADPH oxidase

To eliminate NADH/NADPH oxidase activity, NADH/NADPH oxidase was purified (Table 2). The oxidase was purified 34.6-fold with a yield of 0.38%. The purified preparation showed a single band on a native PAGE (Fig. 1.3 A).

TABLE 1.2. Purification of NADH/NADPH oxidase from *B. subtilis* IFO 3007

Fractionation step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	2940	23500	8.00	1	100
DEAE-Sephacel	404	9390	23.3	2.91	40.0
$(NH_4)_2SO_4$	377	9170	24.4	3.04	39.0
Butyl-Toyopearl	24.2	3280	136	17.0	14.0
Gigapite	7.20	1170	163	20.4	4.99
Toyopearl HW-65	2.30	414	180	22.6	1.76
1st Mono Q	0.630	158	250	31.3	0.674
2nd Mono Q	0.320	89.7	277	34.6	0.382

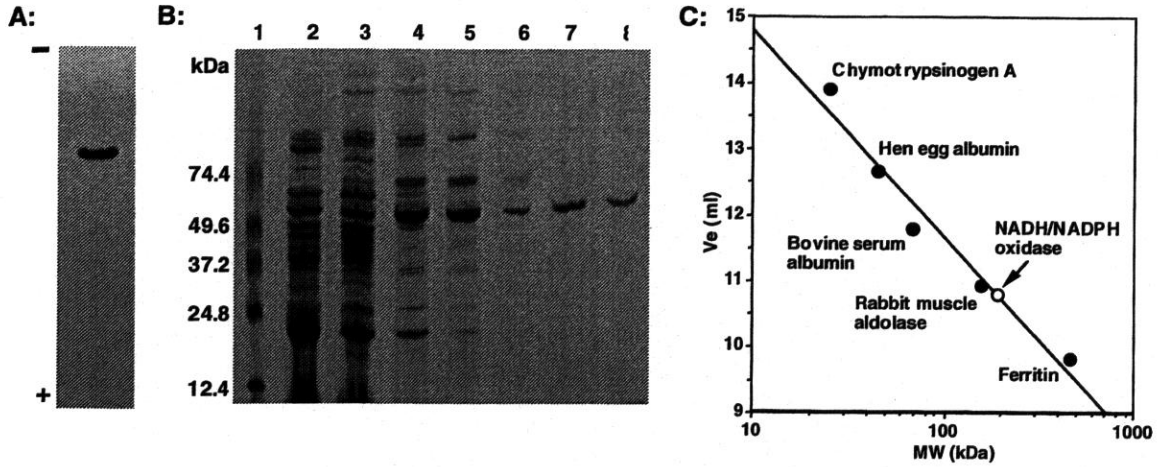

FIG. 1.3. (A) Native PAGE. (B) SDS-PAGE of NADH/NADPH oxidase. Lane 1, marker proteins; lane 2, crude extract; lane 3, after DEAE-Sephacel; lane 4, Butyl-Toyopearl; lane 5, Gigapite; lane 6, Toyopearl HW-65; lane 7, 1st Mono Q; lane 8, 2nd Mono Q. (C) Molecular mass estimation with Superose 12 HR 10/30.

Determination of N-terminal and internal amino-acid sequences

The N-terminal portion of the purified oxidase was sequenced. The 23 residues determined were *N*- M-V-L-D-A-N-I-K-A-Q-L-N-Q-Y-M-Q-L-I-E-N-D-D-V. The amino acid sequences of internal portions obtained were T-X-I-L-S-T-G-A-R-X-R-N-V-N-V-P, M-G-S-G-A-D-A-S-E-F-A-D-K-E-P-F, D-Y-D-I-D-V-M-N-L-Q-R-A-K and G-V-A-Y-E-P. As a result of BLAST search on *B. subtilis* sequence data of SubtiList (http://genolist.pasteur.fr/SubtiList/), the purified enzyme was found to be the product of *ahpF*.

Enzymatic characteristics of NADH/NADPH oxidase

The molecular mass of native NADH/NADPH oxidase was estimated to be about 190 kDa by Superose 12 (Fig. 1.3 C). The purified NADH/NADPH oxidase showed a single band and migrated to a molecular mass position of about 50 kDa on SDS-PAGE (Fig. 1.3 B). These results indicated that the oxidase is probably a homotetramer. The absorption spectrum of the purified enzyme had maxima at 280, 380, and 448 nm and a shoulder at

474 nm (Fig. 1.4). The visible spectrum is characteristic of a flavoprotein. The purified oxidase could use either NADH or NADPH as the reducing agent for O_2. The enzyme also had the ability to reduce other electron acceptors such as menadione, cytochrome *c* and 2,6-dichloroindophenol (data not shown).

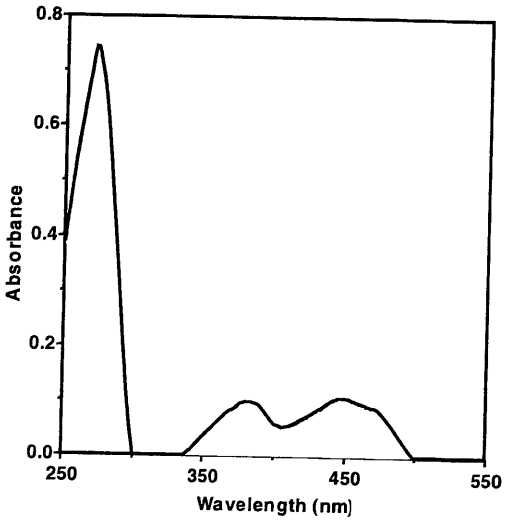

FIG. 1.4. UV-visible absorption spectrum of NADH/NADPH oxidase.

SECTION 3. Investigation of GA-forming activity of GADH

In section 2, it was found that NADH/NADPH oxidase was encoded by *ahpF*. In this section, *ahpF* of *B. subtilis* CRK6000 (Moriya *et al,* 1990) was disrupted to eliminate the coexisting high NADH/NADPH oxidase activity. Then, the GA-forming activity of GADH in *ahpF* disruptant was investigated.

MATERIALS AND METHODS

Materials

Following enzymes were used in this study: restriction endonucleases (Nippom gene, Tokyo, Japan); *TaKaRa Ex Taq* and DNA Ligation Kit Ver. 2 (Takara shuzo Co., Tokyo, Japan).

DNA manipulations

DNA manipulations were done as described by Sambrook *et al.* (1989).

Disruption of *ahpF*

For disruption of *ahpF*, a 410-bp internal fragment of *ahpF* was amplified from *B. subtilis* CRK6000 chromosomal DNA by PCR with primers AHPF1F (5'-GCCG*AAGCTT*CTCAAAGTTAGCGCAGG-3') carrying a *Hin*dIII restriction site (shown in italic) and AHPF1R (5'CGC*GGATCC*TTTGTATGCTGCACCGTC-3') carrying a *Bam*HI site (shown in italic). The PCR product was digested with *Hin*dIII and *Bam*HI in parallel with a pMutin1. After phenol/chloroform treatments, the PCR product and the plasmid were ligated to create pAF111, and was used to transform competent cells of *E. coli* C600. The recombinant plasmid, pAF111, was extracted and introduced into *B. subtilis* CRK6000 by transformation, a single crossover with selection for erythromycin-resistance (0.3 μg/ml), yielding a strain BSAF11. The integration of a

single copy of the plasmids was confirmed by PCR with primers AHPF 2F (5'-ACAAA GGCTGGCATGACAGC-3') and AHPF 2R (5'-CTTCTCAAGGCGTTTCGCAC-3').

RESULTS

Disruption of *ahpF* and purification of GADH

ahpF of *B. subtilis* CRK6000 was disrupted with the pMutin1 integrational vector as described in MATERIALS and METHODS. It was found that the crude extract of the *ahpF* disruptant had lower NADH/NADPH oxidase activity (1.92 U/mg) than that of the wild type-strain (8.00 U/mg). The crude extract of the *ahpF* disruptant was applied to a DEAE-Sephacel column as described in section 1. The elution pattern and the distribution of the oxidizing activity of GADH were identical to those of *B. subtilis* IFO 3007 (Fig. 4). There was no NADH/NADPH oxidase activity in either the first or the second fraction. However, the glycolate-reducing activity (GA-forming activity) was not detected in either the first or the second active GADH fraction (data not shown).

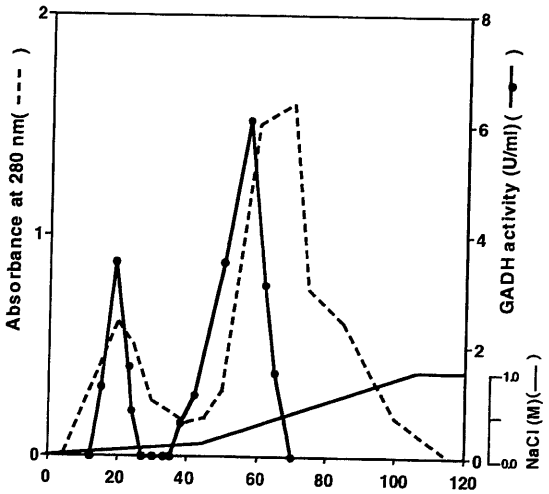

FIG. 1.5. DEAE-Sephacel column chromatography of GADH from *B. subtilis* CRK6000.

SECTION 4. Investigation of glyoxal reductase

In section 3, it was found that the GA-forming activity of GADH was not detected in *B. subtilis*. Therefore, in this section, the other GA-forming enzyme (Fig. 1.6), glyoxal reductase (GR) was investigated.

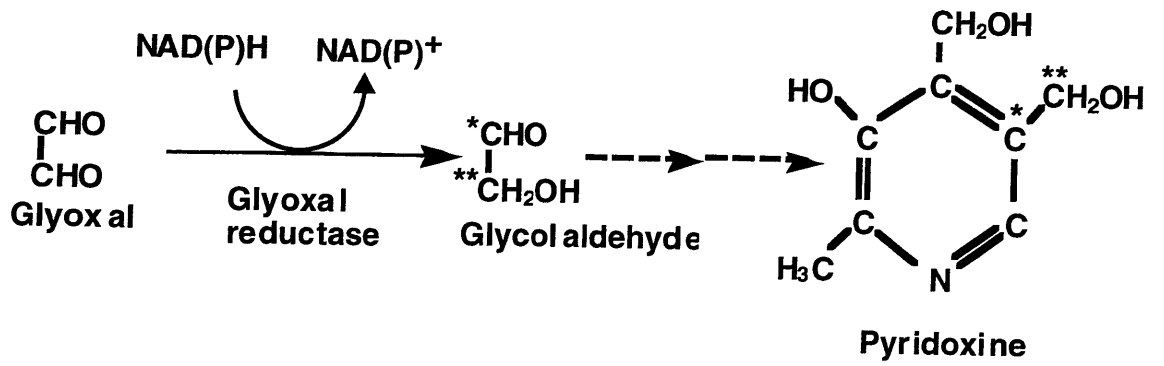

Fig. 1.6 Glycolaldehyde-forming route in *E. coli*

MATERIALS AND METHODS

Enzyme assay

GR activity was assayed at 37℃ for 5 min in the reaction mixture containing 69 μmol of glyoxal, 0.9 μmol of NADPH, 300 μmol of KPB and the enzyme in a total volume of 3.0 ml.

Purification of GR

GR was purified using the following procedure: the crude extract was applied to a DEAE-Sephacel column and eluted with a linear gradient of NaCl from 0.1 to 1.0 M in KPB. Fractions having high enzyme activity were pooled and brought to 50% saturation with solid $(NH_4)_2SO_4$. Precipitates formed were removed by centrifugation, and the resulting supernatant was applied to a Butyl-Toyopearl 650S column and eluted by a

linear gradient of $(NH_4)_2SO_4$ from 50% to 0% saturation in KPB. The pooled active fractions were desalted and concentrated, then applied to a Hiload 16/60 Superdex 75 prep grade column (Amersham Pharmacia), eluted with the same buffer, and active fractions were pooled.

N-terminal and internal amino-acid sequencing

The protein sample was run on a precast gel (Pagesheet SDS GRD 4-20%; Biomate Co., Tokyo) using Pagebox (Bio-Rad). Then proteins were blotted onto Immobilon-P (Millipore Corp., Bedford, MA) and stained with coomassie brilliant blue. The N-terminal amino-acid sequence of protein bands was determined using the 492 protein sequencer (PE-Applied Biosystems). A high-molecular weight calibration kit (Amersham Pharmacia) was used as the molecular weight standards for SDS-PAGE.

Disruption of *yvgN*

yvgN was disrupted by the same method as *ahpF* disruption. Primers used for the amplification of a 307-bp internal fragment were YVGN1F (5'-GCCG*AAGCTT*GAAAT GCCTTGGTTCGGTC-3') carrying a *Hin*dIII restriction site (shown in italic) and YVGN1R (5'-CGC*GGATCC*TATCTTTGCCAGGCCAATG-3') carrying a *Bam*HI site (shown in italic). Primers used for confirmation of the integration of a ingle copy of the plasmids were YVGN2F (5'-GTGCCAACAAGTTTAAAAGATACTG-3') and YVGN2R (5'-GATGAACTTGGAAATTGCTGAC-3').

Construction and purification of glutathione S-transferase (GST)-YvgN fusion protein

yvgN was amplified from *B. subtilis* CRK6000 chromosomal DNA by PCR with primers YVGN1 (5'-CGC*GGATCC*GTGCCAACACGTTTAAAAGATAC-3') carrying a *Bam*HI restriction site (shown in italic) and YVGN2 (5'-CCG*CTCGAG*CGGTTAAAA

CAGAAGCTCATCAGG-3') carrying a *Xho*I site (shown in italic). The PCR product was digested with *Bam*HI and *Xho*I in parallel with a pGEX-4T-1. The digested PCR product and the pGEX-4T-1 were ligated, and then transformed in *E. coli* JM109. *E. coli* JM109 harboring the pGEX-4T-1-*yvgN* was cultivated in Luria-Bertani medium (containing 50 μg/ml ampicillin) at 37°C. After the addition of isopropyl 1-thio-β-D-galactoside to a final concentration of 1.0 mM, the culture was further incubated. The cells were harvested and disrupted with 0.1-mm glass beads using a Mini-Beadbeater (Biospec Products). Cell debris was removed by a centrifugation. The resulting supernatant was loaded onto a Glutathione Sepharose 4B column (Amersham Pharmacia Biotech), and GST-YvgN fusion protein was eluted.

Growth analysis of *yvgN* disruptant

B. subtilis CRK6000 was grown at 37°C in Spizizen minimal medium supplemented with 0.05 mM adenine sulfate dihydrate, 0.05 mM guanosine, 0.5 mM L-methionine and 0.5 mM L-histidine (SMM).

Quantification of B_6 production

The amount of B_6 in the culture was quantified in a microbiological assay using *Saccharomyces carlsbergensis* ATCC 9080 (Tani, 1983). The wild-type strain and the *yvgN* disruptant of *B. subtilis* were cultured in Spizizen minimal medium until the early stationary phase. H_2SO_4 was added to 0.055 N to those cultures, autoclaved at 121°C for 240 min, and then centrifuged at 12,000 × *g* for 15 min. The supernatants of 0.1-0.25 ml were used for samples. Those samples were added into test tubes containing 2 ml of B_6 assay medium (Nissei, Japan), *S. carlsbergenesis* cells and 1.75-1.90 ml of sterilized distilled water. Tubes were cultivated at 30°C for 18 h, and the optical density at 600 nm was determined.

RESULTS

Purification of GR from *ahpF* disruptant of *B. subtilis* CRK6000

The reduction of glyoxal by GR lead to the formation of GA (Fig. 1.6). GR was partially purified 440-fold with a yield of 12.9% (Table 1.3). As a result, a major band and three minor bands were detected by SDS-PAGE (Fig. 1.7 A).

TABLE 1.3. Purification of GR from *ahpF* disruptant of *B. subtilis* CRK6000

Fractionation step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	1360	3830	2.82	1	100
DEAE-Sephacel	207	3970	19.2	6.81	104
$(NH_4)_2SO_4$	164	3400	20.7	7.34	88.8
Butyl-Toyopearl	2.81	1890	673	239	49.3
Hiload 16/60 Superdex 75	0.396	493	1240	440	12.9

Determination of N-terminal amino-acid sequence of purified proteins

The N-terminal portions of the purified proteins were sequenced. The 30 residues of the major protein (indicated by arrow 2, Fig. 5 A) determined were *N*- P-T-S-L-K-D-T-V-K-L-H-N-G-V-E-M-P-P-F-G-L-G-V-F-K-V-E-N-N-N. The 10 residues of the protein (indicated by arrow 1) were *N*-K-Y-I-G-Y-V-G-T-Y-K. The 32 residues of the protein (indicated by arrow 3) were *N*-M-R-E-V-K-D-N-L-A-Q-I-N-L-E-I-N-E-H-R-E-K-S-N-I-I-P-Q-X-N-V-I-A, and the 20 residues of the protein (indicated by arrow 4) were *N*-A-V-Q-Y-E-T-K-A-T-N-V-G-I-R-K-G-H-V-Q-T. As a result of BLAST search on *B. subtilis* sequence data of SubtiList, the purified major protein (indicated by arrow 2) was found to

be the product of *yvgN*, and the protein indicated by arrow 1 was the product of *ykgB*. The other proteins indicated by arrows 3 and 4 could not be identified.

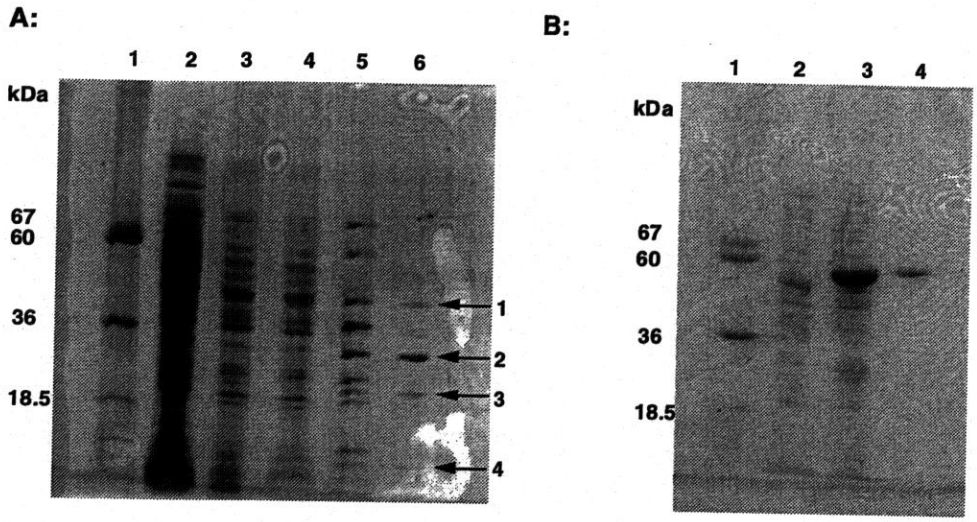

FIG. 1.7. (A) SDS-PAGE of purified glyoxal reductase. Lane 1, marker proteins; lane 2, crude extract; lane 3, DEAE-Sephacel fraction; lane 4, $(NH_4)_2SO_4$ fractionation; lane 5, Butyl-Toyopearl; lane 6, Hiload Superdex 75 prep grade. (B) SDS-PAGE of GST-YvgN fusion protein. Lane 1, marker protein; lane 2, crude extract from strain JM 109; lane 3, crude extract from strain JM 109 harboring pGEX-4T-1-*yvgN*; lane 4, purified GST-YvgN fusion protein.

Expression of *yvgN*

It was very likely that *yvgN* encoded GR because YvgN belongs to the aldo-keto reductase superfamily. Therefore, *yvgN* was cloned into pGEX-4T-1, and expressed as GST-YvgN fusion protein in *E. coli* JM109. GST-YvgN fusion protein was purified (Fig. 1.7), and its properties were determined. YvgN showed GR activity, and also showed relatively high affinity to DL-glyceraldehyde, DL-glyceraldehyde 3-phosphate and methylglyoxal (Table 1.4).

TABLE 1.4. Substrate specificity of GST-YvgN fusion protein

Substrate	Relative activity (%)
Glyoxal	100
Methylglyoxal	98
Glyoxylate	13
DL-Glyceraldehyde	65
DL-Glyceraldehyde 3-phosphate	52
Acetaldehyde	0
Formaldehyde	0
Glycolaldehyde	0

Property of the *yvgN* disruptant

To examine the relationship of *yvgN* to B_6 biosynthesis, *yvgN* of *B. subtilis* CRK6000 was disrupted with the pMutin1 integrational vector, and the properties of the *yvgN* disruptant were examined. When the *yvgN* disruptant was cultivated in SMM (Spizizen minimal medium), the *yvgN* disruptant grew as well as the wild-type strain (Fig. 1.8 A), and did not require B_6. It was also found that the *yvgN* disruptant retained its ability to synthesize the same amount of B_6 as the wild-type strain (Fig. 1.8 B). The *yvgN* disruptant did not show any distinctive phenotype.

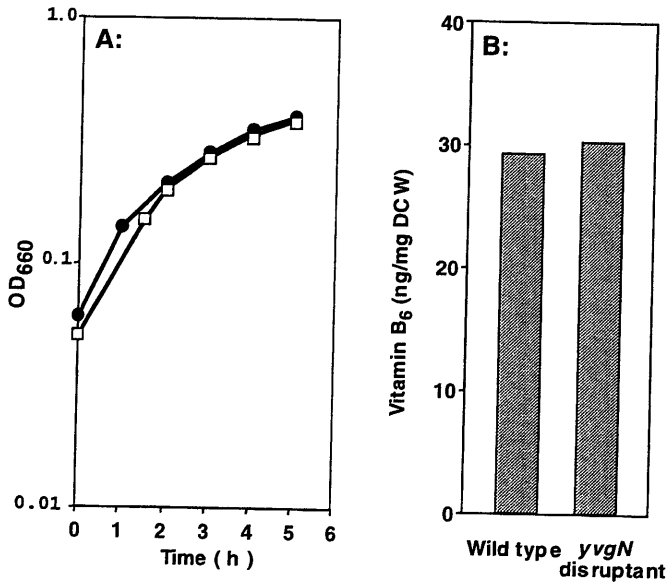

FIG. 1.8. (A) Amounts of B_6 synthesized by *B. subtilis* CRK600 wild type and *yvgN* disruptant. (B) Growth curves of wild type (●) and *yvgN* disruptant(□).

DISCUSSION

In this chapter, the glycolaldehyde-forming route in B_6 biosynthesis in *B. subtilis* was investigated. Firstly, the author purified GADH to investigate its reducing (GA-forming) activity. The oxidizing activity of GADH was detected, but its reducing activity could not be measured because of the coexisting NADH/NADPH oxidase activity. These two enzymes were not separated by DEAE-Sephacel and Butyl-Toyopearl column chromatography. Because NADH/NADPH oxidase might have higher affinity to NADH/NADPH, the reducing activity of GADH was never expected to be determined as long as NADH/NADPH oxidase was present. To eliminate the coexisting oxidase, the author first purified the oxidase from the IFO 3007 strain, and found that NADH/NADPH oxidase was encoded by *ahpF,* of which possible function was previously reported (Hartford, 1994). Then, the *ahpF* disruptant of *B. subtilis* CRK6000 was constructed. The crude extract of the *ahpF* disruptant still had low NADH/NADPH oxidase activity. This finding suggests the presence of the isozymes of NADH/NADPH oxidase, although they did not interfere with the assay for the reducing activity of GADH. Therefore, the author did not try to eliminate the isozymes. GADH of the *ahpF* disruptant was purified by DEAE-Sephacel column chromatography. The elution pattern was the same as that of the IFO 3007 strain, and NADH/NADPH oxidase activity was not detected in either the first or the second active fraction of GADH. However, the reducing (GA-forming) activity of GADH was not detected, either. Therefore, the author concluded that GA is not formed by the reduction of glycolate with GADH in *B. subtilis.* The author also concluded that unlike *E. coli,* GADH is not involved in B_6 biosynthesis because GADH does not synthesize GA, and therefore, did not investigate GADH further. During the

purification of GADH, yields were much higher than 100% (Table 1.1). The reason was assumed to be that inhibitors of GADH were removed during the purification.

The author, next, investigated the other GA-forming route (Fig. 1.6). Partially purified GR was found to be encoded by *yvgN*. The author confirmed that GST-YvgN fusion protein had GR activity. It was found that the *yvgN* disruptant did not require B_6 and retained its ability to synthesize B_6 (Fig. 1.8 A, B). Under the conditions for enzymatic assay, no GR activity was detected. At this point, the presence of isozymes of GR, which have very weak GR activity, cannot be denied. From the above results, however, it was obvious that GR encoded by *yvgN* is not involved in B_6 biosynthesis in *B. subtilis*. To auther's knowledge, there is no report on the function of YvgN. In this study, the author found that YvgN reduced glyoxal and some other compounds (Table 1.4).

SUMMARY

The author concluded that GADH does not synthesize GA, and is therefore not involved in B_6 biosynthesis in *B. subtilis*. It was also concluded that another possible GA-forming enzyme, GR is not involved in B_6 biosynthesis. The results of this chapter demonstrate that the GA-forming route in *B. subtilis* is different from the route in *E. coli*, and GA is possibly not a precursor of B_6 in *B. subtilis*.

CHPTER II

serC is Involved in Vitamin B_6 Biosynthesis in *Escherichia coli* but not in *Bacillus subtilis*

INTRODUCTION

From genetic studies, genes such as *pdxA, pdxB, serC* and *pdxJ* were shown to be possibly involved in vitamin B_6 (B_6) biosynthetic pathway in *Escherichia coli.* A biosynthetic pathway of *E. coli* was proposed (Lam & Winkler, 1990). On the pathway, PdxB, SerC (phosphoserine aminotransferase; EC 2.6.1.52) and other enzymes lead erythrose 4-phosphate to 4-hydroxy-L-threonine (Lam & Winkler, 1990). Then, 4-hydroxy-L-threonine undergoes a decarboxylation, and forms pyridoxine (PN) with 1-deoxy-D-xylulose (Fig. 2).

4-Hydroxy-L-threonine is synthesized by a pathway parallel to the biosynthetic pathway of L-serine (Fig. 2.1). SerA, SerB and SerC are involved in serine biosynthesis, and PdxB, SerC and another enzyme are involved in 4-hydroxy-L-threonine synthesis. SerC catalyzes the transamination of both L-serine and 4-hydroxy-L-threonine synthesis.

In this chapter, the author focused on *serC,* in knowing whether the same pathway as *E. coli* exists in *B. subtilis. serC* disruptants of *E. coli* and *Bacillus subtilis* were constructed, and compared to each other.

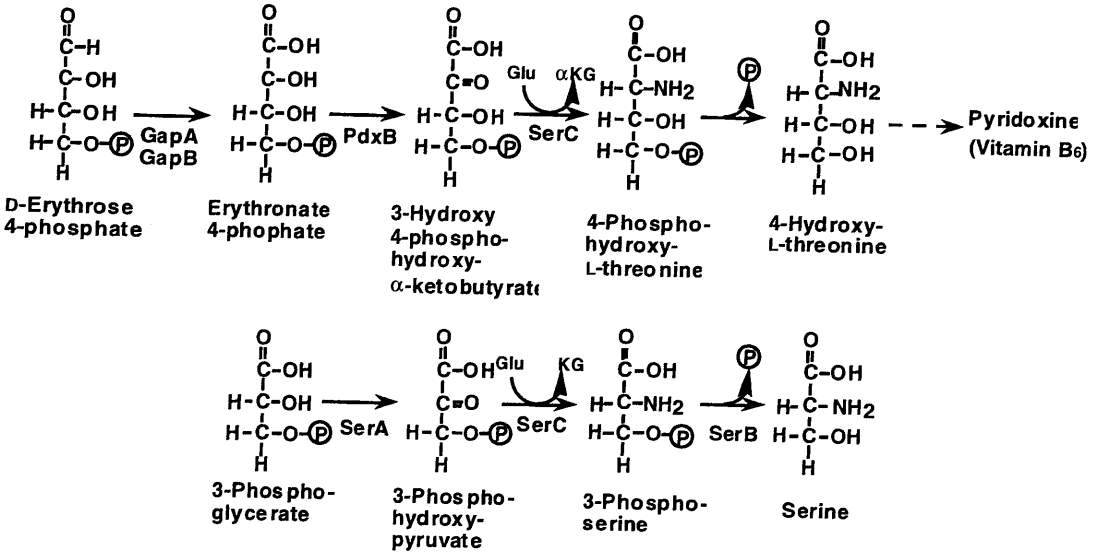

Fig. 2.1. Serine and 4-hydroxy-L-thronine Biosynthetic Pathway of *E. coli.*

MATERIALS AND METHODS

Materials

Following enzymes were used in this study: restriction endonucleases (Nippom gene, Tokyo, Japan); *TaKaRa Ex Taq* and DNA Ligation Kit Ver. 2 (Takara shuzo Co., Tokyo, Japan). GA was purchased from Aldrich Chem. Co. (Milwaukee, WI). Other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan), otherwise specified.

Organisms and culture conditions

The strains and plasmids used are shown in Table 2.1. *B. subtilis* was grown at 37°C in Spizizen minimal medium supplemented with 0.05 mM adenine sulfate dihydrate, 0.05 mM guanosine, 0.5 mM L-methionine and 0.5 mM L-histidine (SMM) or Antibiotic medium 3 (PAB) (Difco Laboratories, Detroit, Mich.). *E. coli* was grown at 37°C in Davis minimal medium supplemented with 0.5 mM shikimic acid, 0.5 mM L-tryptophan, 0.5 mM L-tyrosine and 0.5 mM L-phenylalanine (DMM) or Luria-Bertani (LB) medium.

For the growth study, L-serine, glycine, GA or PN was added to each minimal medium. When it was necessary, vitamin-free casamino acids (Difco Laboratories) were added to the minimal medium at a final concentration of 0.01%. Antibiotics were used at the following concentrations: 0.3 μg erythromycin/ml for the *serC* disruptant of *B. subtilis* and 50 μg kanamycin/ml for the *serC* disruptant of *E. coli.*

DNA manipulations

DNA manipulations were done as described by Sambrook *et al.* (1989).

Construction of *serC* disruptants

The *serC* disruptant of in *B. subtilis*, was constructed as described in Chapter I. The internal fragment of *serC* was amplified from *B. subtilis* CRK6000 chromosomal DNA by PCR with primers SERC1F (5'-GCCG*AAGCTT*TGTTATGGAGCTTTCCCACC-3') carrying a *Hin*dIII restriction site (shown in italic) and SERC1R (5'-CGC*GGATCC*AA TATCGCTGGACATGTCGG-3') carrying a *Bam*HI site (shown in italic). Primers used for the confirmation of integration of a single copy of the plasmids were SERC2F (5'-AG CGCTGCCACTGGAAGTTC-3') and SERC2R (5'-AATGTCGGCGGAGTGTTGTAG AG-3').

For the disruption of *serC* in *E. coli*, a 487-bp internal fragment of *serC* was amplified from the chromosomal DNA of *E. coli* K-12 W3110 by PCR with primers SERC1 (5'-GC CGCTCAAATCTTCAATTTTAGTTCTGG-3') and SERC2 (5'-CTGGCGTTTCGTCGA TGG-3'). A pBEN66 was digested with *Eco*RV, and a single 3'-T overhang was added to each end. Then, resultant plasmid and the PCR product were ligated to create the plasmid pBES11, and was used to transform competent cells of *E. coli* JM109. PBES11 was extracted, and its partial fragment was amplified with primers PBEN+ (5'-CGGTG AAAACCTCTGACAGC -3') and PBEN- (5'-TTTGCTGGCCTTTTGCTCAG -3'). Resultant PCR product (containing Km and *Not*I site at each end, and not containing Amp and origin) was digested with *Not*I, then self-ligated to create the circular plasmid, pBFL31. Resultant plasmid, pBFL31 was introduced into *E. coli* K-12 W3110 by

transformation, a single crossover with selection for kanamycin-resistance (50 μg/ml), yielding a strain ECS021. The integration of sa ingle copy of the plasmids was confirmed by PCR with primers SERC1 and KM2 (5'-GAAGGAGAAAACTCACCGA G-3'), and also confirmed by kanamycin-resistance and ampicillin-sensitive phenotype.

Quantitative analysis of B_6

The amount of B_6 in the culture was quantified in a microbiological assay using *Saccharomyces carlsbergensis* ATCC 9080 as described in Chapter I.

β-Galactosidase assay

β-Galactosidase assays were done by the method of Miller (1972). *B. subtilis* was grown in SMM. Cell samples of 1.0 ml were taken, pelleted by centrifugation, washed with physiological saline, and frozen overnight at -20℃. After cells were suspended in 0.6 ml of Z buffer, 2 drops of chloroform and 1 drop of 0.1% sodium dodecyl sulfate were added, and vortexed. Samples were incubated at 28℃ for 10 min, and then 0.2 ml of 2-nitrophenyl-β-D-galactopyranoside (4 mg/ml) was added. Reaction mixtures were incubated at 28℃ for 30 min, and then 0.3 ml of 1 M $NaCO_3$ was added and vortexed to stop the reaction. Samples were then pelleted at 1,2000 × *g* for 15 min, and the optical density at 420 nm of the supernatant was determined. Miller units are (1,000 × OD_{420})/(time × volume of the cell sample × OD_{600})

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description	References or source
E. coli K-12		
W3110	*E. coli* K-12 prototroph	Beckmann (1972)
JM109	recA1 endA1 gyrA96 thi hsdr17 sepE44 relA1Δ (lac-proAB)/F' [traD36 proAB$^+$ lac Iq lacZΔM15]	Yanisch-Perron *et al.* (1985)
C600	F$^-$ e14$^-$ (McrA$^-$) *thr*-1 *leuB*6 *thi*-1 *lacY*1 *sepE*44 *rfbD1 fhuA21*	Raleigh *et al.* (1998)
ECS021	W3110 *serC*ΩpBFL31(Km^R)	This work
B. subtilis		
CRK6000	*purA16 metB5 hisA3 guaB*	Moriya *et al.* (1998)
BSS117	CRK6000 *serC*ΩpHS011 (Em^R)	This work
Plasmids		
pMUTIN1	Ap^R (*E. coli*), Em^R (*B. subtilis*)	Vagner *et al.* (1998)
pHS011	pMUTIN1 containing part of *serC* (Ap^R, Em^R)	This work
pBEN66	Ap^R, Km^R	H. Mori, unpublished
pBES11	pBEN66 containing part of *serC* (Ap^R, Km^R)	This work
pBFL31	pBEN66 containing part of *serC* (Km^R)	This work

RESULTS

Construction of *serC* disruptants of *B. subtilis* and *E. coli*

serC genes in *B. subtilis* and *E. coli* were disrupted with the pMutin1 and the pBEN66, respectively, as described in MATERIAIS and METHODS. Disruptions of *serC* were shown in Fig. 2.2 and 2.3. In the *serC* disruptant of *B. subtilis* (BSS117), a transcriptional fusion was constructed between the *serC* promoter and the reporter *LacZ* gene.

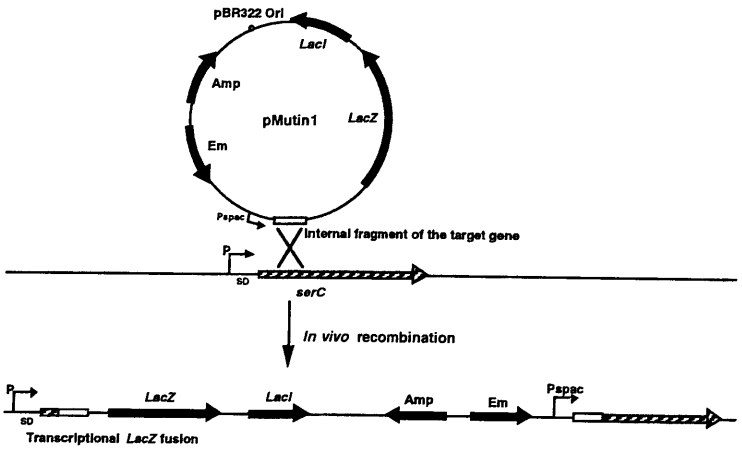

Fig. 2.2. Integration of pMutin1 into *serC* in *B.subtilis*. *serC* is indicated as hatched box . White box corresponds to internal segment of *serC*.

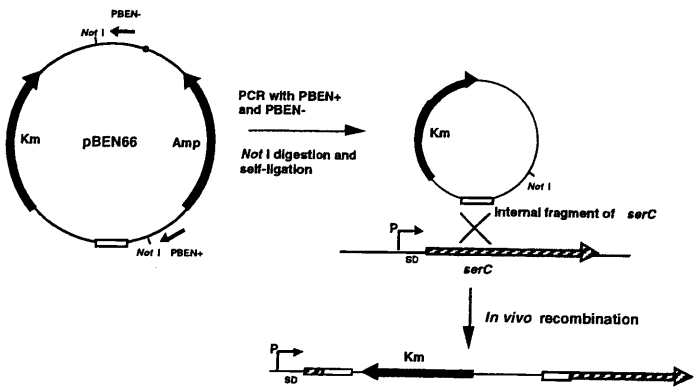

Fig. 2.3. Integration of pBEN66 into *serC* in *E.coli*. *serC* is indicated as hatched box . White box corresponds to internal segment of *serC*.

Analyses of growth requirements

Wild-type and the *serC* disruptant of *B. subtilis* (BSS117) were cultured in SMM to investigate their growth requirements. Cells from overnight culture on PAB plates were inoculated into 5 ml of SMM supplemented with 0.01% casamino acids and incubated until the middle-exponential phase. Cells were collected by centrifugation and resuspended in 1 ml of SMM. Resuspended cells were inoculated into 5.0 ml of SMM supplemented with 1.5 mM L-serine, 0.1 mM PN, 0.1 mM GA and/or 0.5 mM glycine, and then incubated at 37°C (Fig. 2.4). When SMM was supplemented with L-serine, the *serC* disruptant grew, but slower than wild-type. Neither PN nor GA affected the growth of the *serC* disruptant or wild-type. When SMM was supplemented with glycine, the *serC* disruptant grew as wild-type did. Even without L-serine, the *serC* disruptant grew well if glycine was supplied.

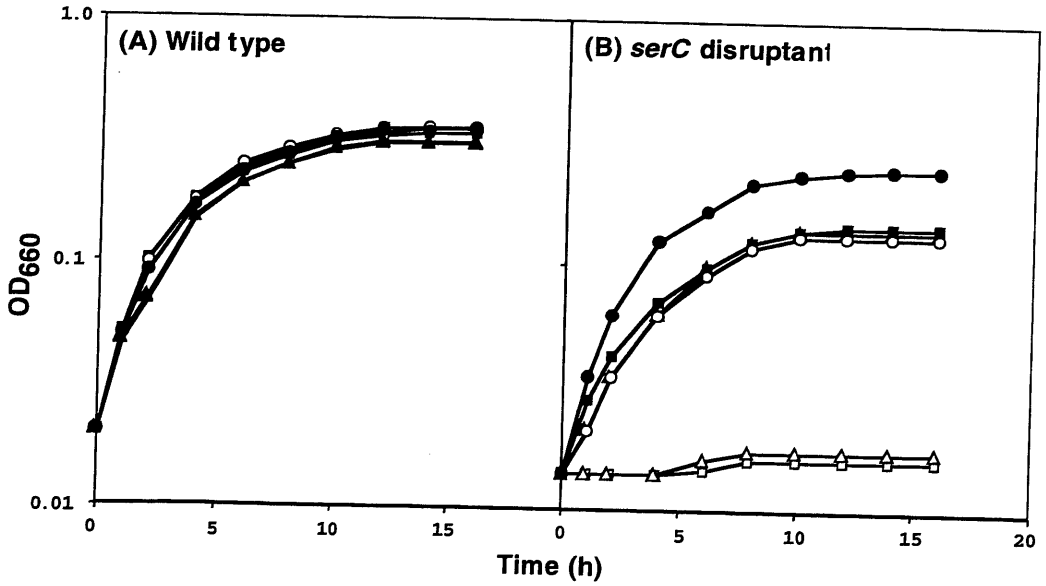

Fig. 2.4. Growth of *B. subtilis* wild type (A) and *serC* disruptant (B) in SMM supplemented with □, none; ■, 1.5 mM L-serine; △, 0.1 mM PN; ▲, 1.5 mM L-serine and 0.1 mM PN; ○, 1.5 mM L-serine and 0.1 mM GA; ●, 0.5 mM glycine.

Growth requirements of the *serC* disruptant of *E. coli* (ECS021) was also examined as the same manner as described above, except for LB plates and DMM were used for the cultivation. Unlike the *serC* disruptant of *B. subtilis*, the *serC* disruptant of *E. coli* could not grow on DMM supplemented with L-serine. The *serC* disruptant of *E. coli* grew only when DMM was supplemented with both L-serine and PN (Fig. 2.5). The *serC* disruptant also grew when DMM was supplemented with L-serine and GA. The *serC* disruptant also grew in DMM supplemented with glycine plus PN or GA (Fig. 2.5).

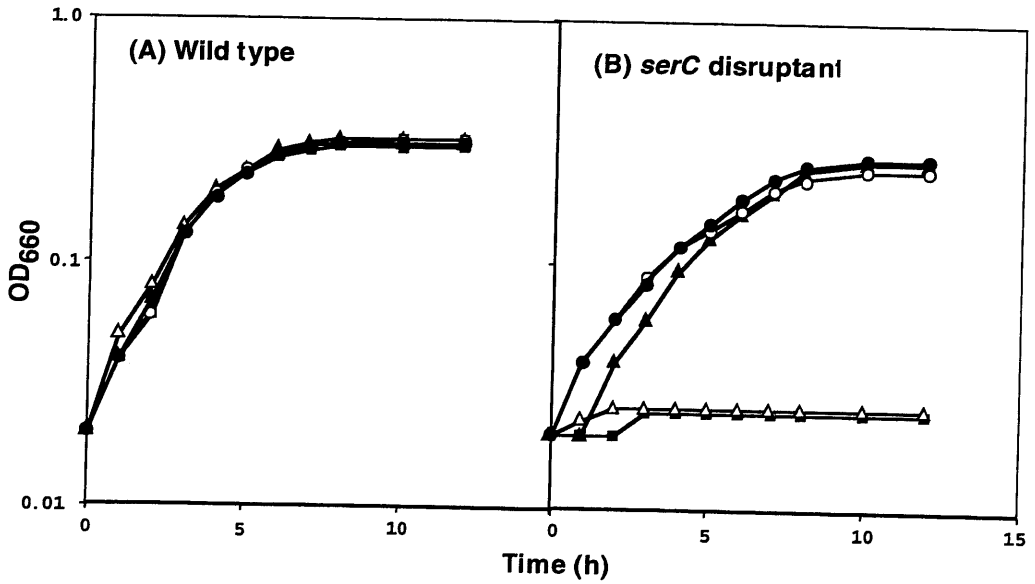

Fig. 2.5. Growth of *E. coli* wild type (A) and *serC* disruptant (B) in DMM supplemented with □,none; ■, 1.5 mM L-serine; △, 0.1 mM PN; ▲, 1.5 mM L-serine and 0.1 mM PN; ○, 1.5 mM L-serine and 0.1 mM GA; ●, 0.5 mM glycine and 0.1 mM PN.

B_6 synthesis of *serC* disruptant of *B. subtilis* and *E. coli*

To determine B_6 amount synthesized, wild-type and the *serC* disruptant of *B. subtilis* and *E. coli* were cultured in minimal media. Cells from overnight culture on PAB or LB

plates were inoculated into 5 ml of SMM supplemented with casamino acids or DMM, and incubated until the middle-exponential phase. The cultures were collected by centrifugation and resuspended in 1 ml of SMM or DMM. Resuspended cells were inoculated into 5.0 ml of SMM or DMM supplemented with L-serine, glycine or GA, and then incubated at 37°C until the early-stationary phase. Then total B_6 amounts were quantified as described in MATERIALS AND METHODS. As shown in Fig. 4, wild-type of *B. subtilis* synthesized 30.2 ng of B_6/mg of cells. The *serC* disruptant synthesized almost the same amount of B_6 (28.1 ng/mg). The addition of GA or glycine to media of wild type and the *serC* disruptant did not affect B_6 synthesis of both strains (Fig. 2.6).

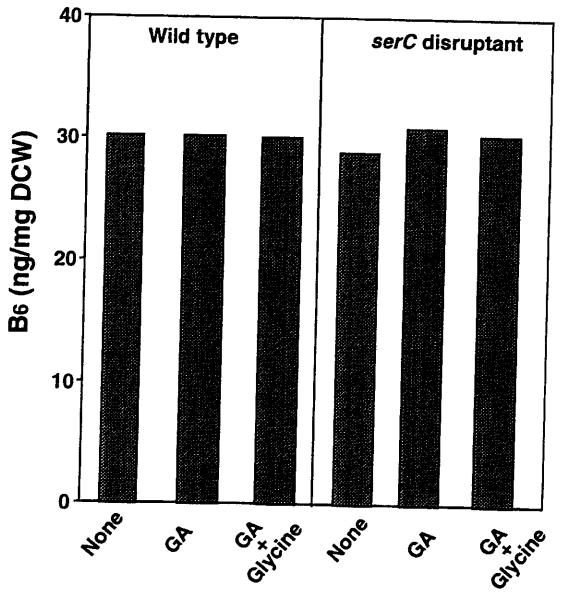

Fig. 2.6. Amount of B6 synthesized by wild type and *serC* disruptant of *B. subtilis* in SMM, SMM supplemented with 0.1 mM GA or SMM supplemented with 0.1 mM GA and 0.5 mM glycine. L-Serine (1.5 mM) was also added to all of SMM.

On the other hand, the *serC* disruptant of *E. coli* clearly lost its ability to synthesize B_6 because the *serC* disruptant could not grew without PN or GA. When the the *serC* disruptant was cultured in DMM supplemented with L-serine and GA, the strain could

grow as well as wild-type (Fig. 2.5). However, B_6 amount synthesized by the *serC* disruptant was less than 20% of that synthesized by wild-type (Fig. 2.7).

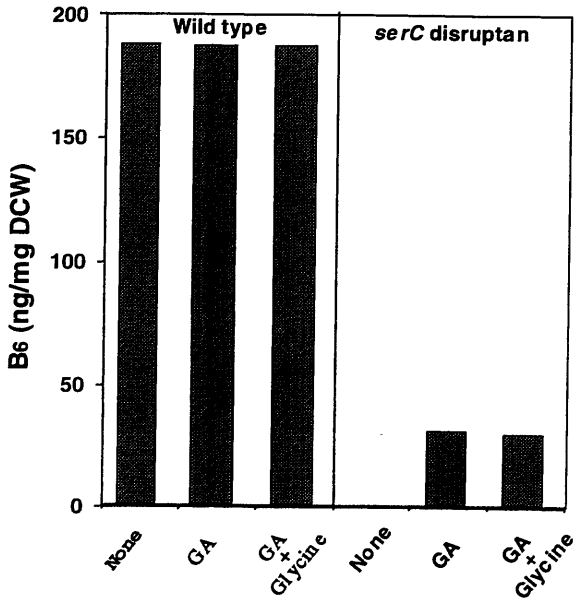

Fig. 2.7. Amount of B6 synthesized by wild type and *serC* disruptant of *E. coli* in DMM, DMM supplemented with 0.1 mM GA or DMM supplemented with 0.1 mM GA and 0.5 mM Glycine. L-Serine (1.5 mM) was also added to all of DMM.

Effect of L-serine, glycine and PN on *serC* transcription in *B. subtilis*

To investigate the effects of L-serine, PN and glycine on the transcription of *serC*, the *serC* disruptant of *B. subtilis* was cultured in SMM, and the expression of *LacZ* reporter gene was measured. Cells from overnight culture on PAB plates were inoculated into 5 ml of SMM supplemented with casamino acids and incubated until the middle-exponential phase. The cultures of 1.0 ml were inoculated into 4.0 ml of SMM (containing 0.5 mM glycine) supplemented with, glycine or PN, and then incubated at 37°C until the late-exponential phase. β-Galactosidase assay was performed as described in MATERIALS and METHODS. At the L-serine concentration of 0.5 mM, no repression or induction of the *serC* transcription was observed (Fig. 2.8). However, higher concentration of L-serine repressed the transcription. L-Serine concentrations at

2.5 mM and 5.0 mM repressed the β-galactosidase activity by about 25% and 30%, respectively. In contrast to L-serine, glycine, which is required by the *serC* disruptant of *B. subtilis*, had no effect on the transcription (Fig. 2.8). Even though 5.0 mM glycine was supplemented to the medium, no repression of the *serC* transcription was observed. The effect of PN, a possible end product of *serC*, on the serC transcription was also examined. However, PN did not affect the β-galactosidase activity (Fig. 2.8).

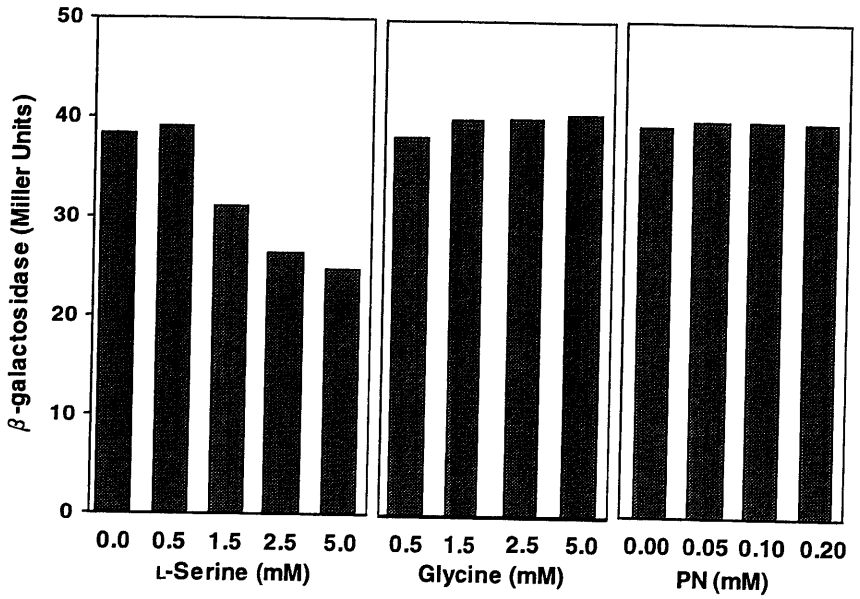

Fig. 2.8. Effects of L-serine, glycine and PN on β -galactosidase activity from *serC* promoter.

DISCUSSION

In this study, the possible involvement of *serC* in B_6 biosynthesis in *B. subtilis* was investigated since *serC* was considered to be involved in B_6 biosynthesis in *E. coli* (Lam & Winkler, 1990). The role of *serC* in *B. subtilis* was investigated from the comparison of the *serC* disruptants of *B. subtilis* and *E. coli.*

The *serC* disruptant of *E. coli* required L-serine plus PN or GA for growth (Fig. 2.5). The growth pattern is consistent with a previous report (Lam & Winkler, 1990). GA was originally found to replace PN in some *E. coli* B_6 auxotrophs (Dempsey *et al.*, 1971; Tani & Dempsey, 1973). On the other hand, it was found that the *serC* disruptant of *B. subtilis* did not require PN. GA had no effect on the growth of the *serC* disruptant of *B. subtilis* (Fig. 2.4). These results lead the conclusion that disruption of *serC* did not convert wild-type of *B. subtilis* to B_6 auxotroph.

When 0.5 mM of glycine was supplemented, the *serC* disruptant of *B. subtilis* grew well as wild-type did (Fig. 2.4). However, when SMM was supplemented with 1.5 mM of L-serine, the *serC* disruptant grew, but not to the full growth (Fig. 2.4). On the other hand, L-serine and glycine had the same effect on the growth of the *serC* disruptant of *E. coli* (Fig. 2.5). L-Threonine could also support the growth of the *serC* disruptant. When L-threonine was added to DMM plus PN, the *serC* disruptant grew very slowly (data not shown), possibly by the catalysis of threonine aldolase (EC 4.1.2.5). The addition of L-threonine to SMM (containing L-serine) improved the growth of the *serC* disruptant of *B. subtilis* to nearly the full growth (data not shown), as previously reported (Vandeyar and Zahler, 1986). However, L-threonine by itself did not support the growth of the *serC* disruptant of *B. subtilis* in SMM.

The *serC* disruptant of *B. subtilis* retained its ability to synthesize almost the same amount of B_6 as synthesized by wild-type (Fig. 2.6). The putative precursor of B_6, 4-hydroxythreonine, might be synthesized from GA and glycin in *E. coli* (Dempsey, 1987). Neither GA nor glycine affected the synthesis of B_6 (Fig. 2.6). It was also found that the

serC disruptant of *E. coli* cultured with GA and L-serine could synthesize much lower amount of B_6 compared than wild-type (Fig. 2.7). Either a difficulty of incorporation of GA into B6 molecule or a deficiency of precursors other than GA in the *serC* disruptant might explain the result. Group I mutant of *E. coli* in which GA replace B_6 for the growth, showed its ability to synthesize the same amount of B_6 as wild-type when cultured with GA (Dempsey *et al.*, 1971; Tani & Dempsey, 1973). Thus, lack of the ability to synthesize a normal amount of B_6 under the presence of GA, is unique to the *serC* disruptant.

The effects of PN, L-serine and glycine on the transcription *serC* were examined using *LacZ* transcriptional fusion in the *serC* disruptant of *B. subtilis* (Fig. 2.2). Results showed that PN and glycine did not affect the transcription *serC*, but excess L-serine did (Fig. 2.8). It is not necessary that PN repress the transcription *serC* even though *serC* is involved in B_6 biosynthesis because other genes for B_6 biosynthesis could be regulated by PN. However, this finding could be one of the evidence to support the prediction that *serC* is not involved in B_6 biosynthesis in *B. subtilis*. Glycine (0.5 mM) was added to SMM for β-galactosidse assay to improve the growth of the *serC* disruptant of *B. subtilis*. Glycine had no effect on the transcription *serC* (Fig. 2.8).

SUMMARY

The results of this chapter demonstrate that *serC* is not directly involved in B_6 biosynthesis, and a pathway involving genes other than *serC* might exist in *B. subtilis*. Recent reports suggested that genes other than *pdx* and *serC* genes are possibly involved in B_6 biosynthesis of *Aspergillus nidulans* and *Cercospora nicotianae*, and different pathways exist (Osmani *et al.*, 1999; Ehrenshaft *et al.*, 1999). In Chapter I, it was suggested that GA is probably not a precursor of B_6 in *B. subtilis*. These results support the prediction that the B_6 biosynthetic pathway in *B. subtilis* is different from that in *E. coli*.

CHAPTER III

Investigation of *tkt* of *Bacillus subtilis* in Relation to Vitamin B_6 Biosynthesis

INTRODUCTION

In *Escherichia coli*, 4-hydroxy-L-threonine is believed to be a direct precursor of vitamin B_6 (B_6). 4-hydroxy-L-threonine is synthesized from D-erythrose 4-phosphate (Fig. 2, Fig. 2.1). Transketolase (Tkt) is involved in D-erythrose 4-phosphate synthesis, and catalyzes reactions in the pentose phosphate pathway, which is one of the routes of intermediary carbohydrate metabolism (Fig. 3.1). In *E. coli*, there are two *tkt* genes, *tkt A* and *tkt B*. When both *tkt A* and *tkt B* were disrupted in *E. coli*, the double disruptant required B_6 (Zhao and Winkler, 1994). Therefore, two *tkt* genes are believed to be involved in B_6 biosynthesis in *E. coli*.

In *B. subtilis*, only one gene is identified as *tkt*. In this chapter, *tkt* was disrupted in *B. subtilis*, and the involvement of *tkt* in B_6 biosynthesis was investigated. General characteristics of the *tkt* disruptant were also described.

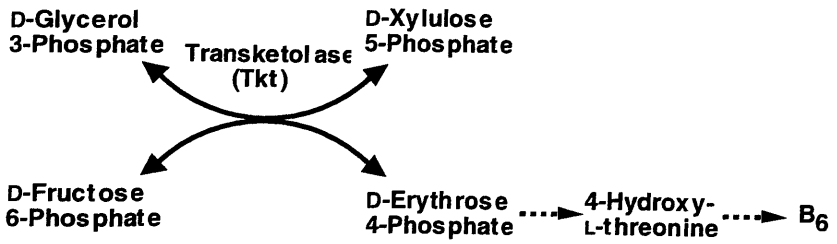

Fig. 3.1. Transketolase transfers a 2-carbon unit from an *alpha*-ketose (a sugar with a carbonyl group at position 2) to an aldose. In the reaction, a 2-carbon unit from the 5-carbon *alpha*-ketose xylulose 5-phosphate is transferred to the 4-carbon aldose erythrose 4-phosphate to make the 6 carbon *alpha*-ketose fructose 6-phosphate. Glyceraldehyde 3-phosphate results from the 3-carbon fragment that is cleaved from xylulose 5-phosphate. Fructose 6-phosphate and glyceraldehyde 3-phosphate (the products of the forward reaction) are an *alpha*-ketose and an aldose and that the reaction is reversible.

MATERIALS AND METHODS

Disruption of *tkt*

tkt was disrupted by a pMutin 1 in *B. subtilis* as described in chapter I and II. Primers used for amplification of the internal fragment of *tkt* was TKT1F (5'- CGC *GGATCC*AGCAAATTCTGGTCACCCAG -3') carrying a *Bam*HI restriction site (shown in italic) and TKT1R (5'- GCCG*AAGCTT*GCTTTCTTCTTTACCAAGCGG -3') carrying a *Hind*III site (shown in italic). Primes used for confirmation of the integration of a single copy of the plasmids were TKT2F (5'-GTAAGGGTACAACA CATAAGGAAGGG-3') and TKT2R (5'-CTTTAACAGCTACAGCGAAATGCTC-3').

Cultural condition

B. subtilis CRK6000 strains were grown at 37°C in Spizizen minimal medium supplemented with 0.05 mM adenine sulfate dihydrate, 0.05 mM guanosine, 0.5 mM L-methionine and 0.5 mM L-histidine (SMM), or Antibiotic medium 3 (PAB) (Difco Laboratories, Detroit, MI). Erythromycin (0.3 μg/ml) was added for the cultivation of the *tkt* disruptant.

Spore formation

B. subtilis strains were inoculated into DS medium, and were incubated at 37°C for 72 h, and cultural samples were taken every 24 h. Cultural samples were divided into two portions, and one portion was heated at 80°C for 15 min. Heated and none-heated cultures were plated on PAB plates, and incubated at 37°C overnight. Then, number of viable cells was counted. Sporulation frequency is expressed as the percentage of heat-resistant spores in the total of both spore and vegetative cells.

RESULTS

Growth properties

Wild- type and the *tkt* disruptant were plated on PAB agar plates and incubated at 37 °C. After 2 days of incubation, the *tkt* disruptant grew very slowly on PAB agar plates, and formed tiny colonies compared to wild-type. Similarly, the *tkt* disruptant grew more poorly than wild-type in liquid PAB medium, but the difference was not significant (Fig. 3.2). The growth properties of the *tkt* disruptant in SMM were examined. Cells from overnight culture on PAB plates were inoculated into 5 ml of SMM supplemented with 0.01% casamino acids and 0.5 mM shikimate. D-Glucose was replaced with 1% D-sorbitol. Then, incubated until the middle-exponential phase. Cells were collected by centrifugation and resuspended in 1 ml of SMM. Resuspended cells were inoculated into 5.0 ml of SMM supplemented with 0.5 mM shikimate, and were grown at 37°C. The *tkt* disruptant did not grow at 37°C in SMM (1% D-glucose was the sole carbon source). When D-glucose was replaced with D-sorbitol as a sole carbon source, The *tkt* disruptant grew (Fig. 3.4-A). The addition of B_6 did not affect the growth of the *tkt* disruptant (Fig. 3.3). Those observations suggested that the *tkt* disruptant required shikimate and an appropriate carbon source, but not B_6.

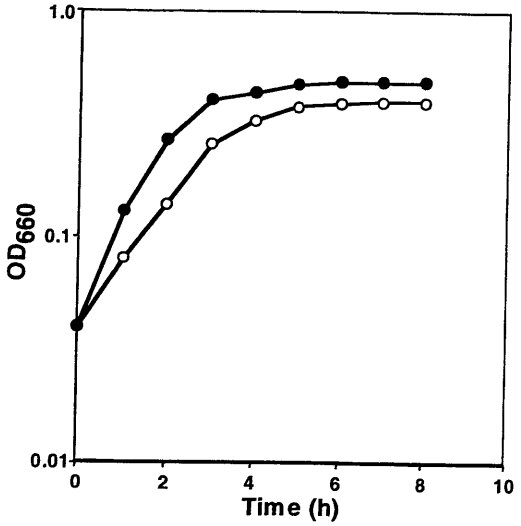

Fig. 3.2. Growth of wild type (●) and *tkt* disruptant (○) of *B. subtilis* in PAB medium.

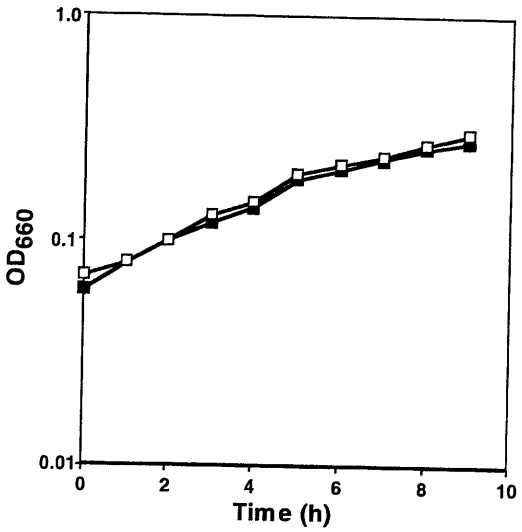

Fig. 3.3. Growth of *tkt* disruptant of *B. subtilis* in SMM supplemented with 0.5 mM shikimate (■), and supplemented with 0.5 mM shikimate and 10 μM PN/PL(□).

Quantification B_6 production

The amount of B_6 in the culture was quantified in a microbiological assay as described in chapter I. Wild-type and the *tkt* disruptant were grown in SMM with 1% D-sorbitol and 0.5 mM shikimate. Cultures were incubated at 37°C for 20 h. The amounts of B_6 in the culture were determined after 1, 4, 8 and 20 h of cultivation (Table 3.1). The amount of B_6 synthesized by wild-type and the *tkt* disruptant were almost the same through the 20 h of cultivation.

Table 3.1. Amounts of B_6 synthesized

Strains	B_6 (ng/mg DCW)			
	Cultivation (h)			
	1	4	8	20
Wild type	1.7	6.6	15	43
tkt disruptant	1.7	8.2	18	36

Utilization of carbon sources

Utilization of carbon sources by the *tkt* disruptant was examined (Fig. 3.4-A). The *tkt* disruptant was grown as the same manner as described above. The *tkt* disruptant was incubated at 37°C in SMM supplemented with shikimate, of which D-glucose was replaced with various carbon sources. As described above, the *tkt* disruptant grew with D-sorbitol, but not with D-glucose. The *tkt* disruptant was also able to utilize D-fructose and glycerol, but not sucrose. However, if D-glucose was added to SMM with D-fructose, the *tkt* disruptant was not able to grow (Fig. 3.4-B). The same result was observed when D-glucose was added to SMM besides glycerol. On the other hand, when D-glucose was supplemented with D-sorbitol, the *tkt* disruptant was able to grow. The growth of the *tkt* disruptant was sensitive to D-glucose when cultivated in SMM with glycelol and D-fructose, but not D-sorbitol. The same effect of D-glucose was not observed on wild-type (Fig. 3.5-A, B).

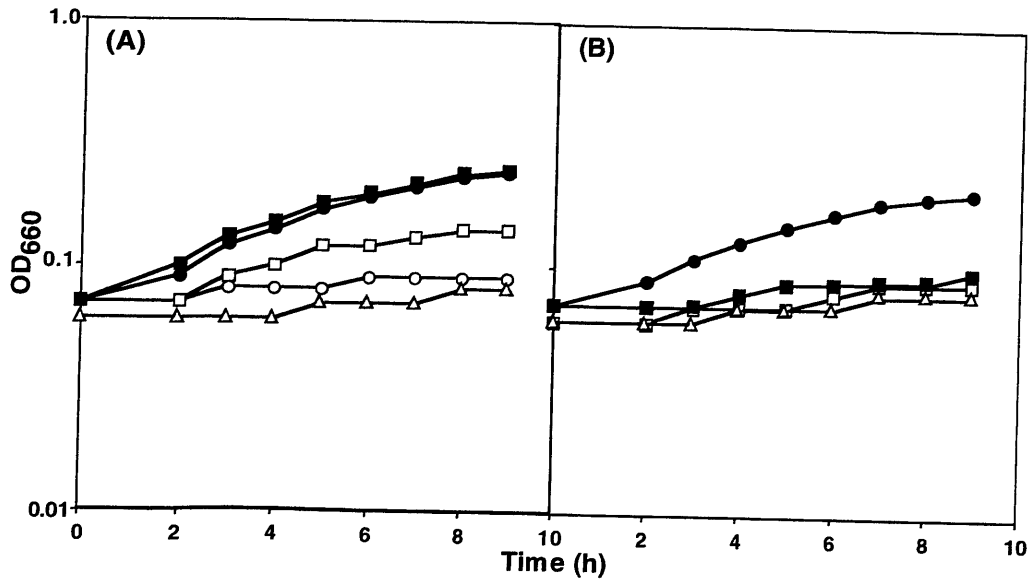

Fig. 3.4. Growth of *B. subtilis tkt* disruptant in SMM supplemented with shikimate. (A) Following sugars (1%) were supplemented as a sole carbon source: ○, D-glucose; ●, D-sorbitol; □, D-fructose; ■, glycerol; △, sucrose. (B) D-Glucose (1%) was added to media in (A).

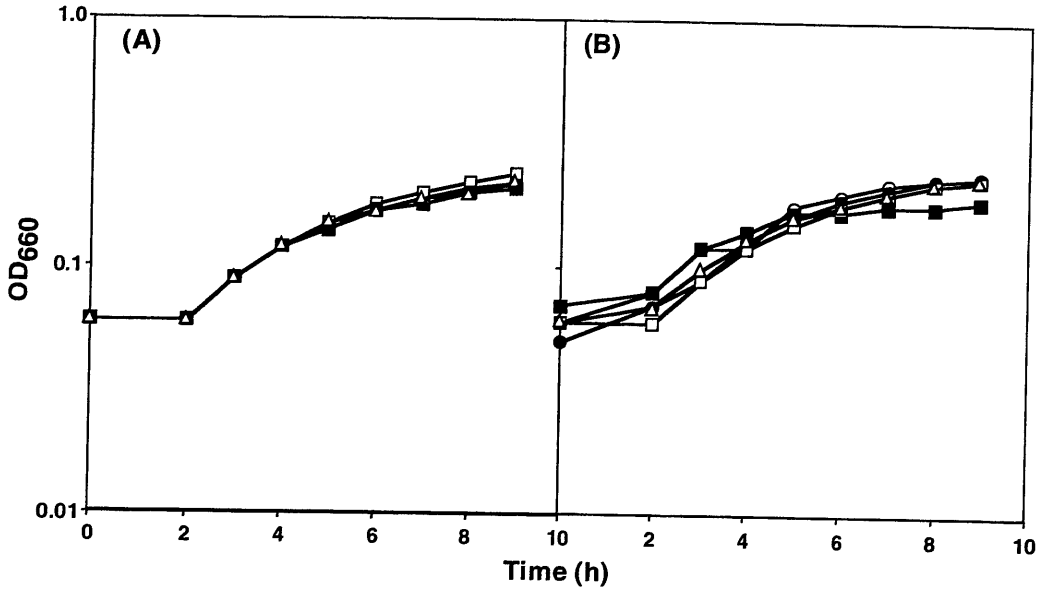

Fig. 3.5. Growth of *B. subtilis* wild type in SMM supplemented with shikimate. (A) Following sugars (1%) were supplemented as a sole carbon source: ○, D-glucose; ●, D-sorbitol; □, D-fructose; ■, glycerol; △, sucrose. (B) D-Glucose (1%) was added to media in (A).

Spore formation

Spore forming ability was examined (Table 3.2). After 24 h of cultivation, the *tkt* disruptant did not form spore at all. After, 48 or 72 h of cultivation, the sporulation frequency was about 50%. In contrast, sporulation frequency of wild-type was 90%-101% through the 72 h of cultivation. The results indicated that Tkt is involved in spore formation.

Table 3.2. Analysis of sporulation

Strains	Sporulation (%)		
	Cultivation (h)		
	24	48	72
Wild type	91	90	101
tkt disruptant	0	50	51

DISCUSSION

Results in this chapter showed *tkt* is not involved in B_6 biosynthesis in *B. subtilis.* The amount of B_6 synthesized by the *tkt* disruptant was almost the same amount as synthesized by wild-type (Table 3.1). After the 20 h of cultivation, wild-type produced a larger amount of B_6. The reason is unknown, but it was difficult to simply compare two strains because the *tkt* disruptant lacks of the pentose phosphate pathway. The entire metabolic pathway could be different in both strains. However, it was obvious that the *tkt* disruptant retained its ability to synthesize B_6. The growth property of the *tkt* disruptant also supports the conclusion that *tkt* is not involved in B_6 biosynthesis (Fig. 3.4). If the *tkt* disruptant lost its ability to synthesize B_6, the disruptant could not grow in SMM even though shikimate and D-sorbitol was supplemented.

In liquid PAB medium, the *tkt* disruptant grew nearly the same rate as wild-type (Fig. 3.2). In contrast, the *tkt* disruptant grew extremely poor, and formed tiny colonies on PAB agar plates. Transketolase mutant is known to be deficient in flagellin (Sasajima and Kumada, 1983). Lack of fllagellin makes the *tkt* disruptant less mobile. Therefore,the *tkt* disruptant might have difficulty to uptake nutrients from agar plate, and formed tiny colonies.

The growth property of *tkt* disruptant might be explained by catabolite repression (Fig. 3.5-A). D-Glucose repressed the enzyme synthesis, which are involved in fructose/glycerol transport and catabolism. Enzymes involved in D-sorbitol transport and catabolism are insensitive to glucose repression (Sasajima and Kumada, 1981).

The reason why the *tkt* disruptant is more sensitive to catabolite repression than wild-type is assumed to be that the cell surface of the *tkt* disruptant was changed (Sasajima and Kumada, 1981).

After 24 h of cultivation in DSM, the *tkt* disruptant did not form spore. The reason might be explain by its slow growth. In DS medium, the *tkt* disruptant grew slower than wild-type. However, even after 48 and 72 h of cultivation, the sporulation frequency was about 50%. The result indicates that *tkt* is involved in spore formation.

In the crude extract of the *tkt* disruptant, no transketolase activity was detected. However, another transketolase, which has extremely low activity, could exist in *B. subtilis*. The second transketolase could complement disrupted *tkt*, and could help B_6 synthesis. *E. coli* has two transketolase, *tktA* and *tktB*. However, it should be noted that no other transketolase homologue is found in the ssequence of *B. subtilis* chromosome.

SUMMARY

This chapter shows that *tkt* is not involved in B_6 biosynthesis in *B. subtilis*. It is difficult to compare B_6 productions of *tkt* diuruptant and wild type because in *tkt* disruptant, one of the main routes of intermediary carbohydrate metabolism, pentose phoshpate cycle does not work. However, results in this chapter clearly demonstrate that *tkt* disruptant retain the ability of B_6 synthesis. It was also emphasized the prediction that B_6 biosynthetic pathway of *B. subtilis* is different from *E. coli*.

CHAPTER IV

Investigation of *yqiE* and *yluB* of *Bacillus subtilis* in Relation to Vitamin B_6 Biosynthesis

INTRODUCTION

In *Escherichia coli*, 1-deoxy-D-xylulose is believed to be a direct precursor of vitamin B_6 (B_6) (Hill *et al.*, 1989, 1996; Kennedy *et al.*, 1995). The compound is an intermediate of non-mevalonate pathway, of which end product, isopentenyl pyrophosphate is a precursor of isoprenoids (Schwender *et al.*, 1996) (Fig. 4.1). The first reaction in the non-mevalonate pathway is the transketolase-like condensation reaction of pyruvate and D-glyceraldehyde to form 1-deoxy-D-xylulose (Sprenger *et al.*, 1997). The reaction is catalyzed by 1-deoxy-D-xylulose synthase (Dxs). Then, 1-deoxy-D-xylulose is further metabolized to 2-*C*-methyl-D-erythritol 4-phosphate by 1-deoxy-D-xylulose reductoisomerase (Dxr) (Takahashi *et al.*, 1998) (Fig. 4.1).

In *Bacillus subtilis*, *yqiE* is a homologue of *dxs*, and *yluB* is a homologue of *dxr*. In this chapter, the author investigated the involvement of *yqiE* and *yluB* to determine whether 1-deoxy-D-xylulose is a precursor of B_6 in *B. subtilis*. Conditional mutants of *yqiE* and *yluB* were constructed, and their abilities of B_6 biosynthesis were examined. *yqxC* and *yluC*, which are downstream genes of *yqiE* and *yluB*, respectively, were also examined (Fig. 4.2).

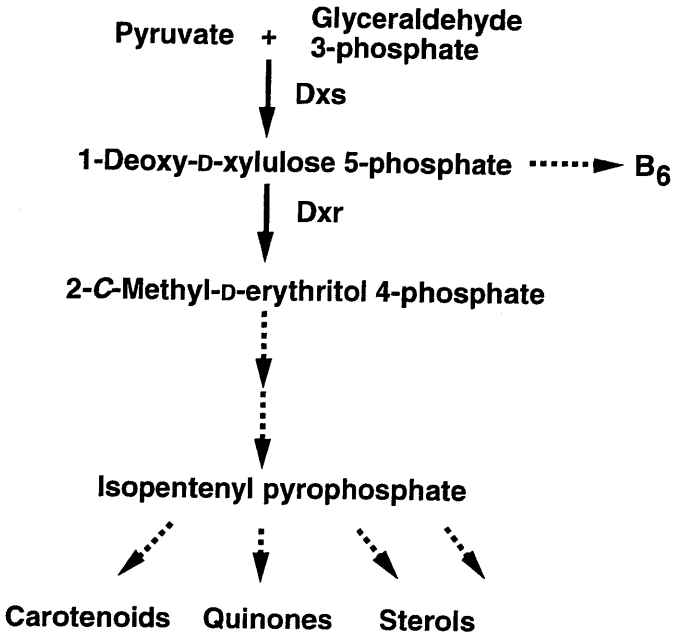

Fig. 4.1. The non-mevaloate pathway. Isopentenyl diphosphate is synthesized from pyruvate and glyceraldehyde 3-phosphate. These two precursors are proposed to condense to give the five-carbon sugar, 1-deoxy-D-xylulose 5-phosphate. 1-Deoxy-D-xylulose 5-phosphate is then converted to isopentenyl pyrophsphate by Dxs, Dxr and other enzymes.

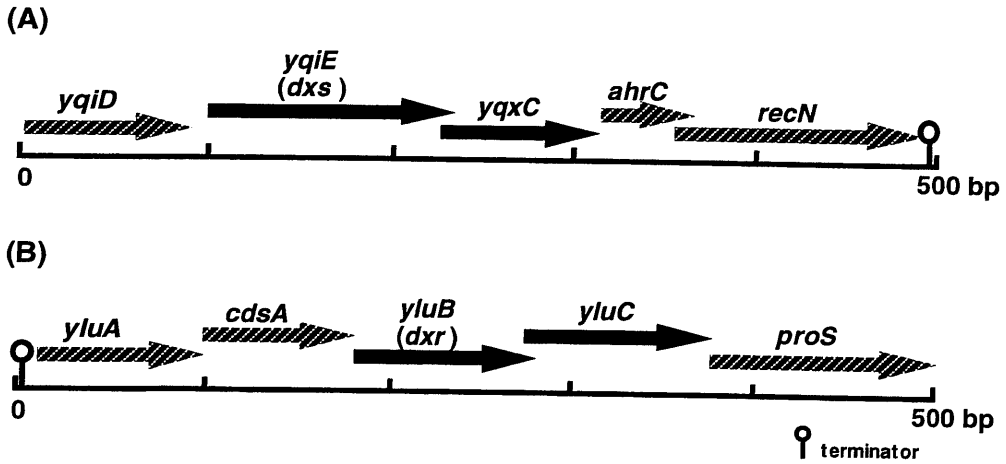

Fig. 4.2. The Orientation of genes aroud (A) *yaiE* and (B) *yluB*.

MATERIALS AND METHODS

Organisms and culture conditions

B. subtilis 168 was grown at 37℃ in Spizizen minimal medium supplemented with 0.5 mM L-tryptophan and 10 mg/L $FeSO_4 \cdot 7H_2O$ (TMM) or Antibiotic medium 3 (PAB) (Difco Laboratories, Detroit, MI). Erythromycin (0.3 μg /ml) was added to medium for disruptants and conditional mutants.

Disruption of genes

Conditional mutants of *yqiE* and *yluB* were constructed by a pMutin 1 in *B. subtilis* 168 as described in chapter I. Primers used in this chapter are summarized in Table 4.1 and Table 4.2. An internal fragment of *yqiE* was amplified with primers, YQIE1F and YQIESD1R. The fragment including the Shine-Dalgarno sequence (SD) of *yqiE* was amplified with YQIESDF and YQIESD1R. The integration of a single copy of the plasmids was confirmed with YQIESD2F and YQIE2R. An internal fragment of *yluB* was amplified with YLUB1F and YULB1R. The fragment including the SD sequence of *yluB* was amplified with YLUBSDF and YULB1R. The integration of a single copy of the plasmids was confirmed with YLUB2F and YLUB2R.

Disruptants of *yqxC* and *yluC* were also constructed by the pMutin 1. An internal fragment of *yqxC* was amplified with primers, YQXC1F and YQXC1R. The integration of a single copy of the plasmids was confirmed with YQIXC2F and YQXC2R. An internal fragment of *yluC* was amplified with YLUC1F and YULC1R. The integration of a single copy of the plasmids was confirmed with YLUC2F and YLUC2R.

Quantitative analysis of B_6

The amount of B_6 was quantified in a microbiological assay as described in Chapter I.

Table 4.1. Primers used for disruption of *yqiE* and *yluB*

Name	Sequence	Restriction site (shown in italic)
YQIE1F	GCCG*AAGCTT*TTATCAATACAGGACCCGTCG	*Hin*dIII
YQIESDF	GCCG*AAGCTT*ATGCTGAAAGTGAGTTGATCCG	*Hin*dIII
YQIE SD1R	CGC*GGATCC*CGTCCGAGCATAGAGTGAATG	*Bam*HI
YQIESD2F	CACGAGGGTATGGTAAAATGTAAGCAG	None
YQIE2R	AAACATTCCGGAGACGAGCATG	None
YLUB1F	GCCG*AAGCTT*GGAGCAACAGGATCAATCG	*Hin*dIII
YLUBSDF	GCCG*AAGCTT*CATGCAGCAATAAGAATAGGAGTAG	*Hin*dIII
YULB1R	CGC*GGATCC*TATATGCCCTGCAGTGACAAG	*Bam*HI
YLUB2F	GCATGGCGGTATATTAGACCG	None
YLUB2R	CTGTTCGCCTTGAAGAGCTTG	None

Table 4.2. Primers used for disruption of *yqxC* and *yluC*

Name	Sequence	Restriction site (shown in italic)
YQXC1F	GCCG*AAGCTT*ATTAGTGGAAAGAGGGCTGG	*Hin*dIII
YQXC SD1R	CGC*GGATCC*TGGAGGAGCCAATATCAATC	*Bam*HI
YQXC2F	TGGACTGACAAAACAGCAGG	None
YQXC2R	AGCTGATTATAGCCTACATCCACAG	None
YLUC1F	GCCG*AAGCTT*TTATTTTCGGAACGCTCG	*Hin*dIII
YULC1R	CGC*GGATCC*TGTTTCGCTGACAGTAAAGC	*Bam*HI
YLUC2F	CTAAATGCGGCAAACGAAG	None
YLUC2R	CAATTGCTTTAATCCGCTGC	None

RESULTS

Construction of disruptant and conditional mutants

Disruptants and conditional mutants of *yqiE* and *yluB* were constructed by the pMutin 1 in *B. subtilis* as described in MATERIALS and METHODS. As a result, conditional mutants of *yqiE* and *yluB*, of which transcriptions could be induced by isopropyl 1-thio-β-D-galactoside (IPTG), were obtained (Fig. 4.3 A, Fig. 4.4 A). Disruptants of *yqxC* and *yluC* were also constructed (Fig. 4.3 B, Fig. 4.4 B). To obtain *yluC* disruptant, the transcriptions of its downstream genes had to be induced by IPTG. *yqxC* disruptant was obtained without the IPTG induction.

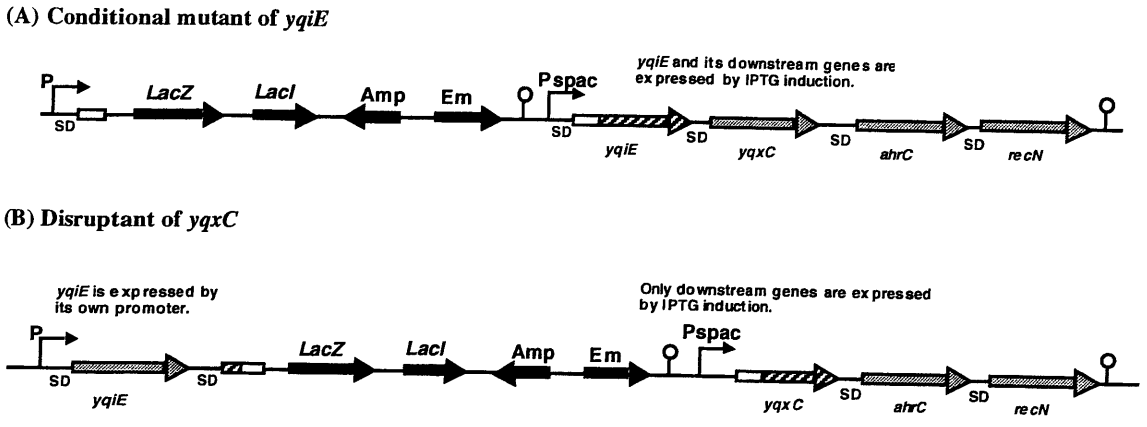

Fig. 4.3. The structure obtained upon integration of the pMutin 1 into (A) *yqiE* or (B) *yqxC* in the *B.subtilis* chromosome.

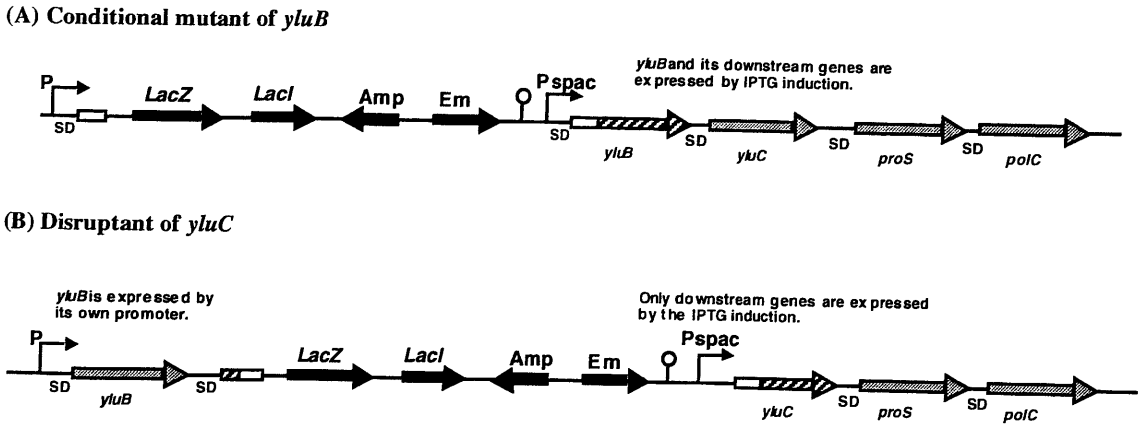

Fig. 4.4. The structure obtained upon integration of the pMutin 1 into (A) *yluB* or (B) *yluC* in the *B.subtilis* chromosome.

B_6 synthesis of the conditional mutant of *yqiE* and the *yqxC* disruptant

Cells from overnight PAB cultures were collected by centrifugation, washed with physiological saline and inoculated into TMM. After 2-4 h of the pre-cultivation, cells were collected by centrifugation and inoculated into 5 ml of TMM. For the pre-cultivation of the conditional mutants of *yqiE*, 0.02 mM of IPTG was added. The indicated amount of IPTG was added to the followed cultivation. Total B_6 amounts were quantified at the indicated times.

Although the *yqiE* mutant grew poorly when no IPTG was added to the medium, the mutant synthesized almost the same amount of B_6 per dry cell weight as the wild-type strain (Fig. 4.5 A B). The addition of IPTG improved the growth of the *yqiE* mutant, but did not affect the B_6 synthesis (Fig. 4.5 A B). The addition of IPTG did not affect the growth of the *yqxC* disruptant (Fig. 4.6 A). The *yqxc* disruptant synthesized almost the same amount of B_6 as the wild-type strain with or without the addition of

IPTG (Fig. 4.6 B), and retained its ability to synthesize B_6. These results indicated that neither *yqiE* nor *yqxC* is involved in B_6 production.

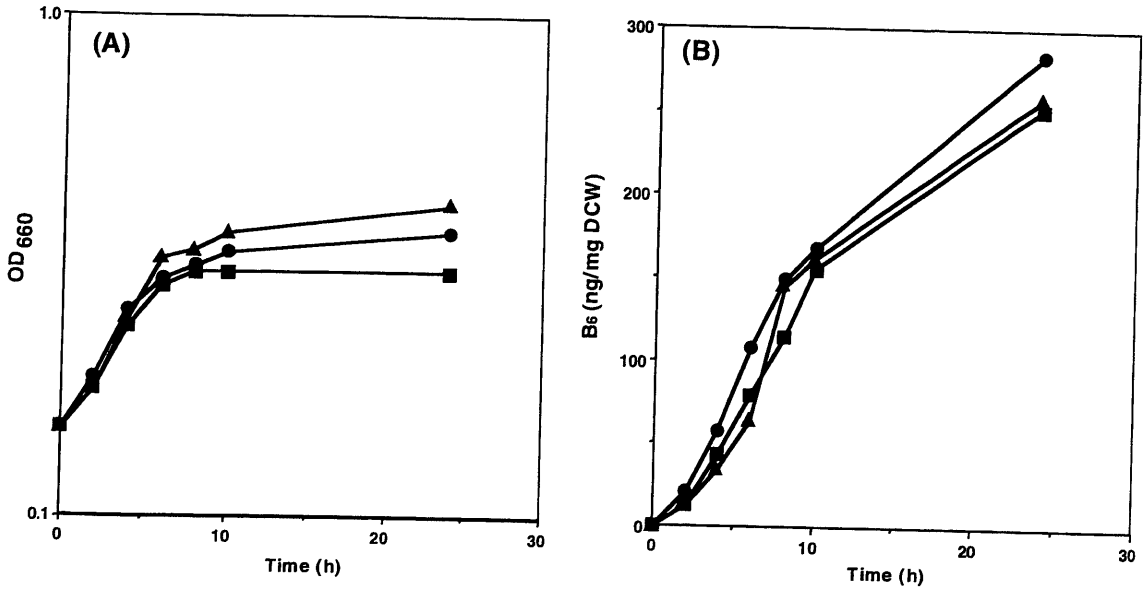

Fig. 4.5. (A) The growth and (B) the B_6 synthesis of the wild-type strain (●), the conditional mutant of *yqiE* without induction (■) or the conditional mutant of *yqiE* induced by 0.1 mM IPTG (▲).

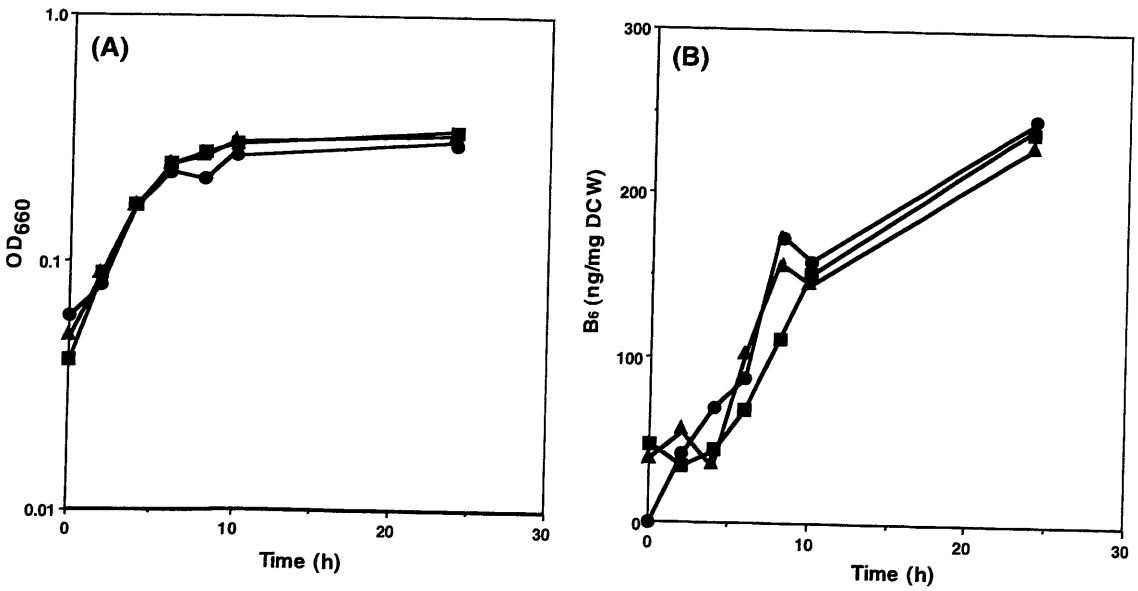

Fig. 4.6. (A) The growth and (B) the B_6 synthesis of the wild-type strain (●), the *yqxC* disruptant without induction (■) or the *yqxC* disruptant induced by 0.1 mM IPTG (▲).

B_6 synthesis of the conditional mutant of *yluB* and the *yluC* disruptant

Cultivations were done as described above, except for no IPTG was added for the pre-cultivation. Unlike the *yqiE* mutant, the conditional mutant of *yluB* grew as well as the wild-type strain without the IPTG induction (Fig. 4.7 A). The induction by IPTG affected neither the growth nor the B_6 synthesis of the conditional mutant of *yluB* (Fig. 4.7 A B). Without the IPTG induction, the *yluC* disruptant grew poorly, but synthesized almost the same amount of B_6 per cells as the wild-type strain (Fig. 4.8 A B). The addition of IPTG improved the growth of the *yluC* disruptnt because the transcriptions of its downstream genes were induced, but did not affect the B_6 synthesis. These results indicated that neither *yluB* nor *yluC* is involved in B_6 synthesis.

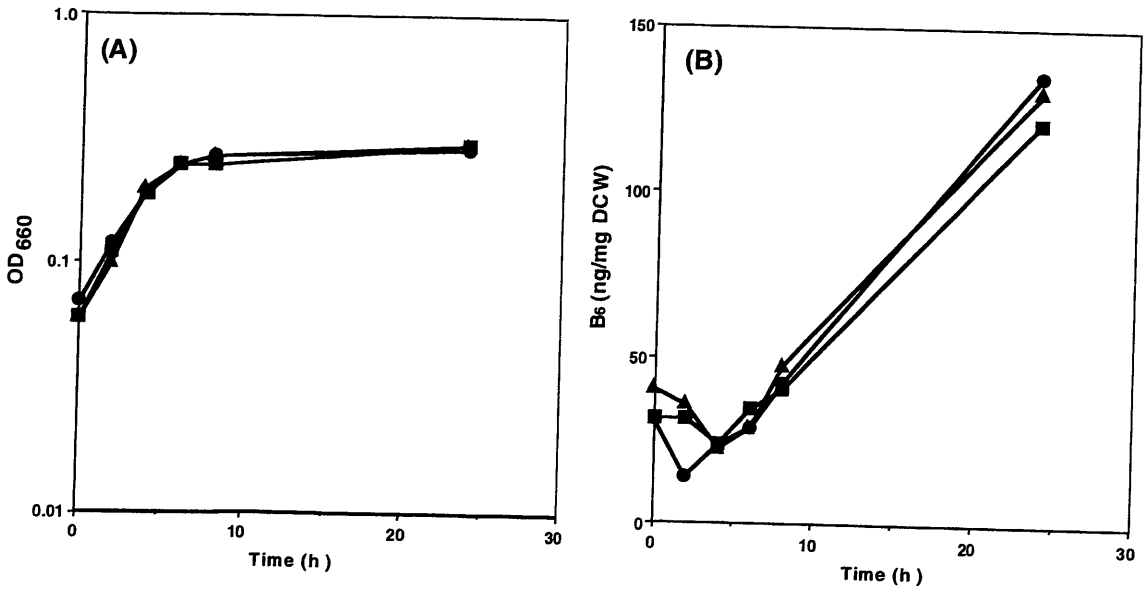

Fig. 4.7. (A) The growth and (B) the B6 synthesis of the wild-type strain (●), the conditional mutant of *yluB* without induction (■) or the conditional mutant of *yluB* induced by 0.1 mM IPTG (▲).

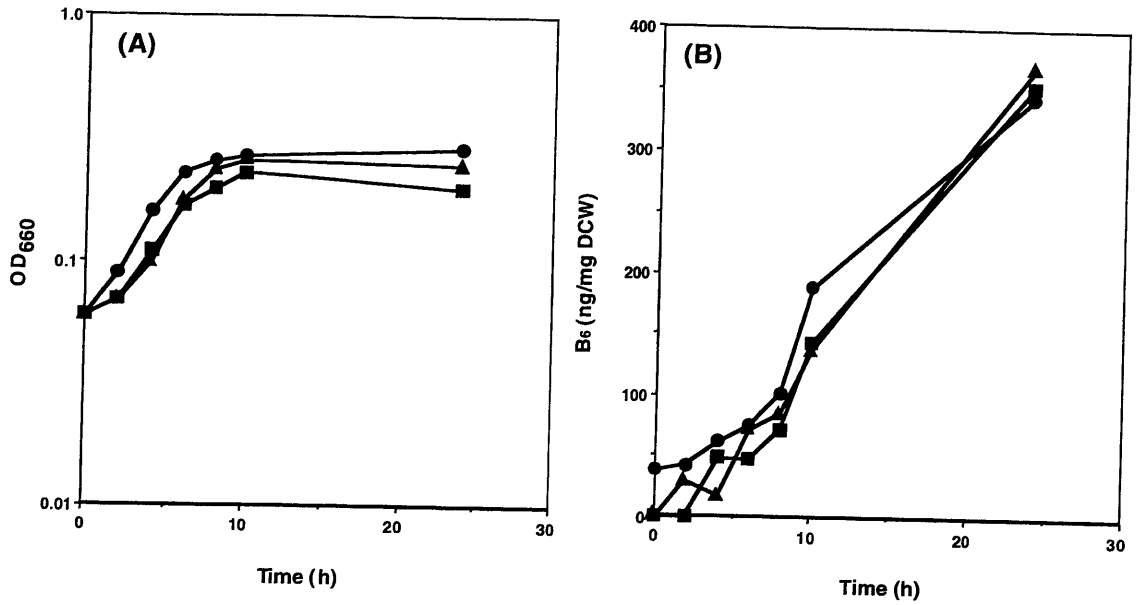

Fig. 4.8. (A) The growth and (B) the B_6 synthesis of the wild-type strain (●), the *yluC* disruptant without induction (■) or the *yluC* disruptant induced by 0.1 mM IPTG (▲).

DISCUSSION

In this chapter, the involvement of *yqiE* and *yluB* in B_6 biosynthesis was investigated. Involvement of *yqxC* and *yluC* was also investigated because the functions of these genes were unknown.

Dxs (YqiE) is considered to be involved in the synthesis of a presursor of B_6, 1-deoxy-D-xylulose in *E. coli*. Therefore, it was predicted that if *yqiE* is disrupted, the disruptant could not synthesize 1-deoxy-D-xylulose and B_6. Results in this chapter showed that *B. subtilis* retained its ability to synthesize B_6 even if *yqiE* is inactivated. Without the induction of IPTG, the *yqiE* mutant synthesized smaller amount of B_6 than the wild-type strain did. The reason is assumed that the mutant grew poorly, and therefore, produced less B_6 than wild-type. The B_6 amount per dry cell weight of the *yqiE* mutant was almost the same as wild-type (Fig. 4 B)

Firstly, the construction of a *yqiE* disruptant was attempted, but was unsuccessful. Even though the transcriptions of its downstream genes were induced by IPTG, a *yqiE* disruptant could not be obtained. The downstream gene of *yqiE*, *yqxC* could be disrupted. Therefore, it is most likely that *yqiE* itself is an essential gene, but not *yqxC*.

The involvement of Dxr (*yluB*) in B_6 biosynthesis was also examined. Dxr converts 1-deoxy-D-xylulose to 2-*C*-methyl-D-erythritol 4-phosphate. Therefore, it was predicted that the inactivation of *yluB* lead to the accumulation of 1-deoxy-D-xylulose, and 1-deoxy-D-xylulose flux through B_6 increases. Alternatively, if the expressional level of *yluB* is increased, 1-deoxy-D-xylulose flux through 2-*C*-methyl-D-

erythritol 4-phosphate is increased, and therefore, the amount of B_6 synthesized is decreased. Results in this chapter showed that alternation of the *yluB* transcription did not affect the B_6 synthesis. The inactivation of its downstream gene, *yluC*, did not affect the B_6 synthesis, neither.

Disruption of *yluB* was firstly attempted. However, no *yluB* disruptant was obtained. Even though the transcriptions of its downstream genes were induced by IPTG, a *yluB* disruptant could not be obtained. Thus, *yluB* is likely an essential gene. The downstream gene of *yluB*, *yluC* itself is not an essential gene. The *yluC* disruptant was obtained if the transcriptions of downstream genes were induced by IPTG. Therefore, its downstream genes, *proS* and/or *polC* are most likely essential genes.

These results indicates that 1-deoxy-D-xylulose is not a precursor of B_6 in *B. subtilis*. If 1-deoxy-D-xylulose is a precursor of B_6 like in *E. coli*, the B_6 synthesis should be decreased in the *yqiE* mutant of *B. subtilis* unless other pathways of 1-deoxy-D-xylulose synthesis exist. In author's knowledge, no 1-deoxy-D-xylulose synthetic pathway other than shown in Fig. 4.1 is reported. Ineffectiveness of alternation of the transcription of *yluB* on B_6 synthesis supports above indication.

SUMMARY

In this chapter, the involvement of *yqiE*, *yluB* and their downstream genes, *yqxC* and *yluC*, in B_6 biosynthesis was investigated. The results of this chapter demonstrate that none of these genes is involved in B_6 biosynthesis in *B. subtilis*. The results suggest that 1-deoxy-D-xylulose might be not the precursor of B_6 in *B. subtilis*. This chapter supports the prediction that B_6 biosynthetic pathway of *B. subtilis* is different from *E. coli*.

CHAPTER V

yaaD and *yaaE* are Involved in Vitamin B_6 Biosynthesis in *Bacillus subtilis*

INTRODUCTION

sor1 gene was identified as a gene involved in resistance of *Cercospora nicotianae* to singlet-oxygen-generating phototoxins, and the growth of *sor1* mutant of *C. nicotianae* was supported by pyridoxine (Ehrenshaft *et al.*, 1999). Similar result was obtained in *Aspergillus nidulans* (Osmani *et al.*, 1999). Mutation of *pyroA*, the homologue of *C. nicotianae sor1,* caused increased sensitivity to singlet oxygen generated by photosensitizer, and addition of PN reversed the toxicity. Both *sor1* and *pyroA* are considered to be the member of highly conserved *SNZ* family. *SNZ* genes were originally identified as genes expressed during the stationary phase in *Saccharomyces cerevisiae,* each of which has another highly conserved gene, *SNO*, upstream (Fuge *et al.*, 1994; Braun *et al.*, 1996; Padilla *et al.*, 1998).

In *Bacillus subtilis, yaaD* was found to be a homologue of *SNZ* family, and *yaaE*, which is located on the downstream of *yaaD*, to be a homologue of *SNO* family (Galperin & Koonin, 1997). However, functions of these genes are currently unknown.

This chapter demonstrates that *yaaD* and *yaaE* are involved in B_6 biosynthesis in *B. subtili. yaaD* and *yaaE* are disrupted in *B. subtilis*, and their requirements were examined. Other characteristics of *yaaD* and *yaaE*were also determined.

MATERIALS AND METHODS

Organisms and culture conditions

The strains and plasmids used are shown in Table 5.1. *B. subtilis* 168 was grown at 37°C in Spizizen minimal medium supplemented with 0.5 mM L-tryptophan and 10 mg/L $FeSO_4 \cdot 7H_2O$ (TMM) or Antibiotic medium 3 (PAB) (Difco Laboratories, Detroit, MI). For *B. subtilis* CRK6000, 0.05 mM adenine sulfate dihydrate, 0.05 mM guanosine, 0.5 mM L-methionine and 0.5 mM L-histidine were added to Spizizen minimal medium (SMM). For the growth study, L (+)-ascorbic acid (AsA), glutathione (GSH), pyridoxine (PN) or pyridoxal (PL) was added to TMM or SMM. For analyses of *yaaD* and *yaaE* transcriptional patterns, DS medium (DSM) was used for cultivation. Erythromycin (0.3 μg /ml) was added to medium for disruptants.

DNA manipulations

DNA manipulations were done as described by Sambrook *et al.* (1989).

Construction of *yaaD* disruptant

Disruptants were constructed as described in Chapter I and II. Primers used for amplification of an internal fragment of *yaaD* YAADF (5'-GCCG*AAGCT*TGTGTA AAACGCGGAATGG-3') carrying a *Hin*dIII restriction site (shown in italic) and YAAD1R (5'-CGC*GGATCC*TGCGAAGCATAGAAGCACC-3') carrying a *Bam*HI site (shown in italic). The integration of single copy of the plasmids was confirmed by PCR with primers YAAD2F (5'-TCAAAGCGCTAAAAACCG-3') and YAAD2R (5'-ATCGCAACTACTTTGCGC-3'). *yaaD* and *yaaE* disruptants of *B. subtilis* 168 were prepared as the same manner as described above, except for a pMutin3 was used instead of a pMutin1.

β-Galactosidase assay

β-Galactosidase assays were done as described in Chapter II.

Table 5.1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description	References or source
E. coli K-12		
C600	F⁻ e14⁻ (McrA⁻) *thr*-1 *leuB6 thi*-1 *lacY*1 *sepE44 rfbD1 fhuA21*	Raleigh *et al.* (1998)
B. subtilis		
CRK6000	*purA16 metB5 hisA3 guaB*	Moriya *et al.* (1998)
168	*trpC2*	N. Ogasawara
BSD111	CRK6000 *yaaD*ΩpHD131 (Em^R)	This work
168YD	168 *yaaD*ΩpMYD (Em^R)	N. Ogasawara
168YE	168 *yaaE*ΩpMYE (Em^R)	N. Ogasawara
Plasmids		
pMUTIN1	Ap^R (*E. coli*), Em^R (*B. subtilis*)	Vagner *et al.* (1998)
pMUTIN3	Ap^R (*E. coli*), Em^R (*B. subtilis*)	Vagner *et al.* (1998)
pHD131	pMUTIN1 containing part of *yaaD*	This work
pMYD	PMUTIN3 containing part of *yaaD*	N. Ogasawara
pMYE	PMUTIN3 containing part of *yaaE*	N. Ogasawara

RESULTS

Construction of *yaaD* and *yaaE* disruptants of *B. subtilis*

yaaD in *B. subtilis* CRK6000 was disrupted with a pMutin1 as described in MATERIALS and METHODS. *yaaD* and *yaaE* of *B. subtilis*168 was disrupted with a pMutin3. Those vectors placed genes downstream of the insert under the control of isopropyl-1-thio-ß-D-galactoside (IPTG)-inducible promoter. In all disruptants, transcriptional fusion was constructed between *yaaD/yaaE* promoter and the reporter *LacZ* gene.

Analyses of growth requirements

To investigate the growth requirement of the *yaaD* disruptant, wild-type and the *yaaD* disruptant (BSD111) of *B. subtilis* CRK6000 were cultured in SMM. Cells from overnight culture on PAB plates were inoculated to 5 ml of SMM supplemented with 0.1 mM PN or PL. IPTG (0.05 mM) was added for the expressions of downstream genes. The cultures were incubated at 37°C, and the optical density at 660 nm was measured at the indicated time (Fig. 5.1). When SMM was supplemented with PN, the *yaaD* disruptant grew, but more slow than wild-type. When SMM was supplemented with PL, the *yaaD* disruptant grew as well as wild-type. Growth requirements of the *yaaD* disruptant (168YD) and the *yaaE* disruptant (168YE) of *B. subtilis* 168 were also examined as the same manner as described above, except TMM was used for the cultivation. The *yaaD* disruptant of *B. subtilis* 168 showed the same growth characteristic as the *yaaD* disruptant of *B. subtilis* CRK6000 (Fig. 5.2). The *yaaD* disruptant grew in TMM supplemented with 0.05 mM PN, but more slow than wild-type. When TMM was supplemented with 0.05 mM PL, the *yaaD* disruptant grew as well as the wild-type strain. The *yaaE* disruptant of *B. subtilis* 168 grew in TMM supplemented with 0.05 mM PN or PL, as well as wild-type. Effects of other antioxidants, AsA and GSH were also examined in the *yaaD* disruptant of *B. subtilis* 168. Neither AsA (0.1 mM) nor GSH (0.1 mM) could replace PN/PL (Fig. 5.3).

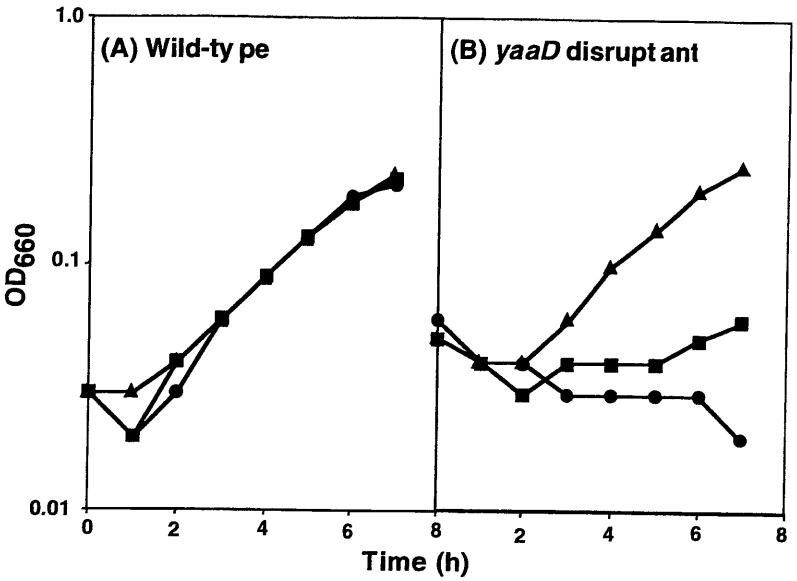

Fig. 5.1. Growth of wild-type (A) and the *yaaD* disruptant (B) of *B. subtilis* CRK6000 in SMM supplemented with ●,none; ■, 0.1 mM PN; and ▲,0.1 mM PL.

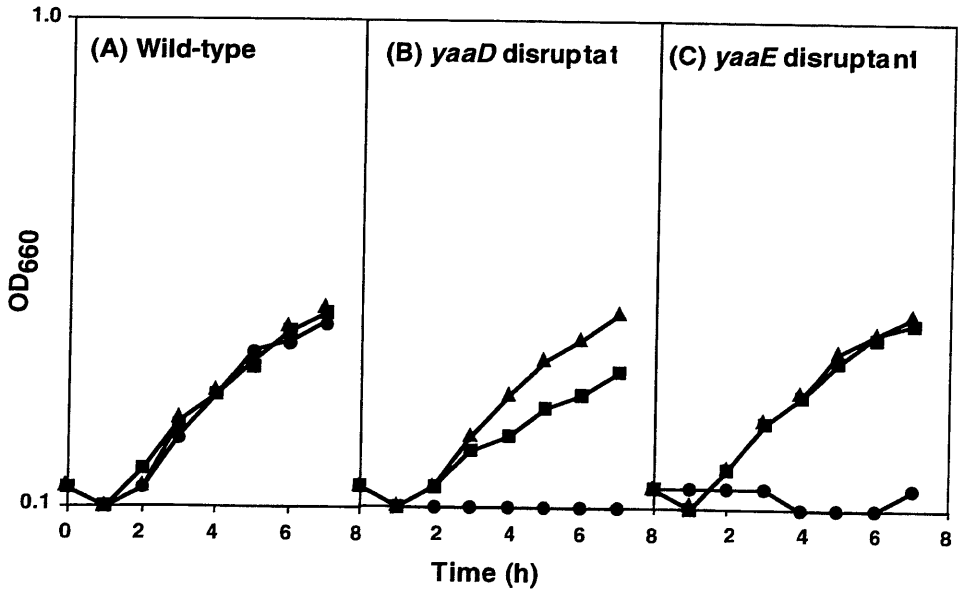

Fig. 5.2. Growth of wild-type (A), the *yaaD* disruptant (B) and the *yaaE* disruptant (C) of *B. subtilis* 168 in TMM supplemented with ●,none; ■, 0.05 mM PN; and ▲, 0.05 mM PL.

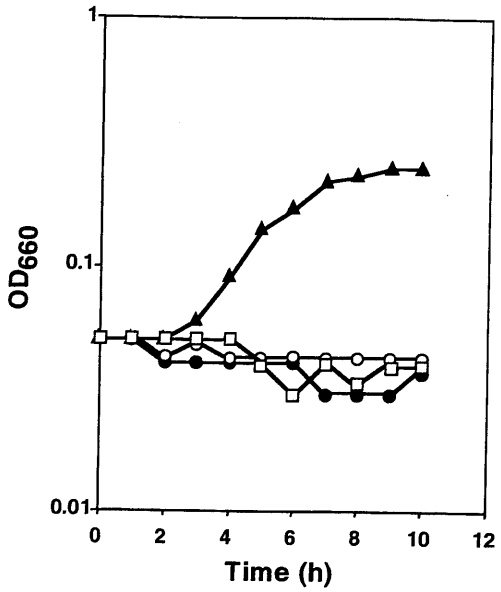

Fig. 5.3. Effects of antioxidants on growth of *yaaD* disruptant; ●,none; ▲, 0.05 mM PL; ○, 0.1 mM AsA and □, 0.1 mM GSH.

Patterns of *yaaD* and *yaaE* transcription

To investigate patterns of the transcriptions of *yaaD* and *yaaE*, the disruptants of *yaaD* and *yaaE* of *B. subtilis* 168 were cultured in DSM, and their expressions of *LacZ* reporter gene were measured. Cells from 5 ml of overnight PAB culture were pelleted by centrifugation, washed with physiological saline, and inoculated into 100 ml of DSM. The cultures were incubated at 37°C until the late-stationary phase. β-Galactosidase activities were measured as described in MATERIALS and METHODS. The β-galactosidase activities of *yaaD* and *yaaE* were the highest at the late-exponential phase and decreased during the stationary phase (Fig. 5.4).

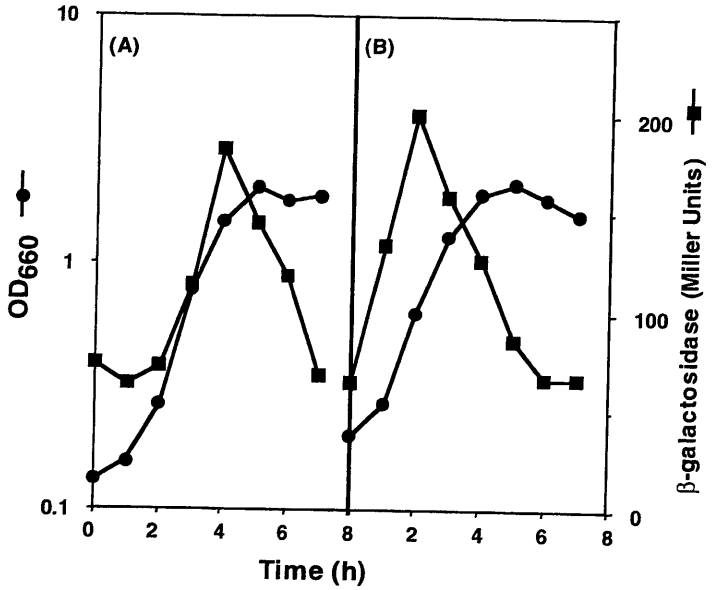

Fig. 5.4. Patterns of *yaaD-LacZ* (A) and *yaaE-LacZ* (B) trascription.

Effects of PN and PL on *yaaD* and *yaaE* transcription

To investigate the effects of PN and PL on the transcription of *yaaD* and *yaaE*, disruptants of *yaaD* and *yaaE* of *B. subtilis* 168 were cultured in TMM, and their expressions of *LacZ* reporter gene was measured. Cells from 5 ml of PAB overnight culture were pelleted by centrifugation, washed with physiological saline, and inoculated into 5 ml of TMM supplemented with PN and PL. The cultures were incubated at 37°C until the late-exponential phase, and the β-galactosidase activities were measured. PN and PL, the possible end products of *yaaD* and *yaaE*, did not affect the β-galactosidase activities of the *yaaD* disruptant or the *yaaE* disruptant (Fig. 5.5).

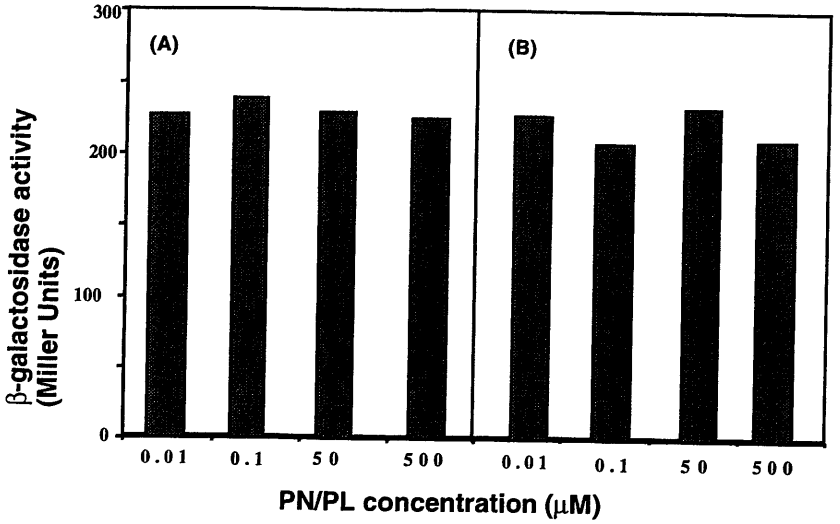

Fig. 5.5. Effects of PN/PL on *yaaD-lacZ* (A) and *yaaE-lacZ* (B) transcription in *B. subtilis*.

DISCUSSION

In this chapter, the involvement of *yaaD* and *yaaE* in B_6 biosynthesis in *B. subtilis* was investigated. The growth requirements of the *yaaD* disruptant of *B. subtilis* CRK6000 (BSD111) and 168 (168YD) were the same. Both strains grew when TMM or SMM was supplemented with PN or PL. The *yaaE* disruptant (168YE) also required PN or PL. However, the growth patterns of the disruptants of *yaaD* and *yaaE* were slightly different. Although PN did not lead the *yaaE* disruptant to the full growth, PL lead the *yaaD* disruptant to the full growth (Fig. 5.1, 5.2). Reproducibility of the growth experiment was confirmed with repeated measurements. Although the result is currently unexplained, it was obvious that the ability to utilize PN is different between the disruptants of *yaaD* and *yaaE*.

Mutation in *pyroA*, the homologue of *C. nicotianae SOR1*, caused increased sensitivity to singlet oxygen-generating phototoxins (Ehrenshaft *et al.*, 1999). Results of this chapter showed that the disruptants of *yaaD* and *yaaE* required PN or PL for their growth, but arose the question of whether these disruptants stopped B_6 biosynthesis or lost their ability to resist to singlet oxygen and required higher amount of B_6 as a singlet-oxygen quencher than wild-type. To address above question, effects of other singlet oxygen quenchers, AsA and GSH, on the growth were examined. These compounds could not support the growth of the *yaaD* disruptant (Fig. 5.3). Therefore, it is most likely that the *yaaD* disruptant required PN or PL because the disruptant lost its ability to synthesize B_6.

SNZ genes were originally identified as genes expressed during the stationary phase in *S. cerevisiae*, each of which has another highly conserved gene, *SNO*, upstream (Padilla *et al.*, 1998). Among *SNZ/SNO* family of *S. cerevisiae*, *SNZ1* and *SNO1* are transcribed during the stationary phase, and are involved in growth arrest (Padilla *et al.*, 1998). However, the transcriptional levels *yaaD* and *yaaE* were the highest at the late-exponential phase, and decreased during the stationary phase (Fig. 5.4), suggesting that

these genes are not directly involved in the growth arrest in *B. subtilis*. It is likely that two genes are cotranscribed, but it is still unknown whether two genes are functionally related to each other or simply catalyze two different reactions.

The downstream gene of *yaaE, serS* (gene for seryl-tRNA synthetase; EC 6.1.1.11), is very likely an essential gene. If *serS* is regulated by the promoter of *yaaD* and *yaaE*, the transcription of *serS* is terminated, and is not transcribed unless induced by IPTG in the *yaaE* disruptant. It was found that the *yaaE* disruptant grew well without IPTG if TMM was supplemented with PL (Data not shown). The result indicates that *serS* is not cotranscribed with *yaaD* and *yaaE,* or *serS* is also transcribed by its own promoter as well as the promoter of *yaaD* and *yaaE.*

It was also investigated if transcriptions of *yaaD* and *yaaE* were repressed by the possible end products, PN or PL because B_6 production is known to be controlled by feedback inhibitions and repression (Dempsey, 1965; Dempsey, 1971b). Since the transcriptions of *yaaD* and *yaaE* were not repressed by either PN or PL (Fig. 5.5), it is unlikely that both genes were regulated by B_6 at the transcriptional level. However, it is still possible that *yaaD* and *yaaE* are controlled with other regulatory systems such as a feedback inhibition.

Until now, no reliable precursor or biosynthetic pathway of B_6 in *B. subtilis* is reported. The functions of YaaD and YaaE are still unknown. Therefore, at this point, it is difficult to discuss precursors or biosynthetic pathways of B_6. Elucidation of the functions of YaaD and YaaE will provide a clue to B_6 biosynthesis of *B. subtilis.*

SUMMARY

In this chapter, it was demonstrated that *yaaD* and *yaaE* are involved in B_6 biosynthesis in *B. subtilis*. It is possible that B_6 biosynthetic pathway of *B. subtilis* is similar to that of *A. nidulans* and *C. nicotianae,* and different from that of *E. coli*. The function of YaaD and YaaE is a further target of B_6 biosynthesis.

GENERAL DISCUSSION

In this thesis, the biosynthesis of vitamin B_6 (B_6) in *Bacillus subtilis* was investigated. Elucidation of biosynthetic pathways usually starts with construction and collection of various auxotrophic mutants. At the beginning of this study, construction of B_6 auxotrophs was attempted with various mutagenesis methods such as ultra violet, nitrosoguanidine, ethyl methanesulfonate and transposon mutagenesis. Approximately 20,000-100,000 colonies were assayed for each method, and approximately 1% of them showed auxotrophy. However, no B_6 auxotroph was derived. The reason is unknown, but one of the reasons is assumed to be that the amount of B_6 required by *B. subtilis* is very low, and therefore, it was very difficult to distinguish B_6 auxotroph from wild-type.

Genes involved in B_6 biosynthesis were searched from genes, of which functions are unknown. Approximately 2,200 disruptants of unknown genes were examined for B_6 auxotrophy. Approximately 1% of the disruptants showed auxotrophy. However, no disruptant showed B_6 auxotrophy, but the disruptants of *yaaD* and *yaaE*.

The author also attempted to identify proteins, of which expressions are repressed by B_6, using two-dimensional (2D) protein electrophoresis. However, no protein on the 2D gel was obviously repressed by B_6. There are approximately 4,100 open reading frames in *B. subtilis*. However, only 100-150 well-separated protein bands could be detected on the 2D gel. Therefore, it was difficult to detect the proteins repressed by B_6 even though the expressions of some proteins are repressed.

In this thesis, therefore, the elucidation of B_6 biosynthesis of *B. subtilis* was primary attempted using information from other microorganisms. Firstly, enzymes and genes involved in B_6 biosynthesis in *Escherichia coli* were investigated. As a result, it was

indicated that the glycolaldehyde-forming route of *B. subtilis* could be different from that of *E. coli*, and glycolaldehyde is not a precursor of B_6 in *B. subtilis*. The author also tried to figure out whether *serC*, *tkt* and *dxs* are involved in B_6 biosynthesis in *B. subtilis*. As a result, it was indicated that these genes involved in B_6 biosynthesis in *E. coli* are not involved in B_6 biosynthesis in *B. subtilis*.

Besides above genes, *pdx* genes (*pdxA, B, H, J, K*) are considered to be involved in B_6 biosynthesis in *E. coli*. In *B. subtilis*, definite homologues of *pdx* genes are not found. Even a homologue of *pdxH* was not found. *pdxH* encodes PNP/PMP oxidase (EC: 1.4.3.5), and is found in *E. coli*, *Haemophilus influenzae*, *Saccharomyces cerevisiae* and *Myxococcus xanthus*. These PdxH have a conserved amino acids sequence, [LIVF]-E-F-W-x (5)-R-L-H-[DE]-R. The conserved sequence was not found in the sequence of *B. subtilis* chromosome. Thus, it is unlikely that *B. subtilis* has *pdxH* and other *pdx* genes.

Next, genes involved in B_6 biosynthesis in other microorganisms than *E. coli* were investigated. *yaaD* is a homologue of genes, which are considered to be involved in B_6 biosynthesis in *Cercospora nicotianae* and *Aspergillus nidulans*. *yaaE* is the downstream gene of *yaaD*. Results of this study suggested that *yaaD* and *yaaE* are involved in B_6 biosynthesis in *B. subtilis*.

yaaD belongs to a highly conserved protein family, Snz family. *yaaE* belongs to another conserved protein family, Sno family. In *B. subtilis* and *H. influenzae*, Snz proteins (YaaD) are immediately followed by Sno protein (YaaE). Snz family is found in all three domains of life, bacteria, archaea and eukaryotes, but not in *E. coli*. Currently, microorganisms can be divided into two groups by the possession of *snz* or *pdxA/J* homologues. Microorganisms with *snz* homologues lack of homologues to the *E. coli* *pdxA/J*, whereas those with homologues to *pdxA/J* lack of *snz* (Ehrenshaft *et al.*, 1999).

B. subtilis has a *snz* homologue (*yaaD*), but not *pdxA/J* homologues. *E. coli* has *pdxA/J*, but not *snz* homologues.

In addition to above grouping, microorganisms were grouped by the difference of the origin of nitrogen atom (Tanaka *et al.*, 2000). In *B. subtilis*, the nitrogen atom of pyridoxine is derived from glutamine. In *E. coli*, the nitrogen atom of pyridoxine is derived from glutamate. In *S. cerevisiae*, the nitrogen atom of PN is derived from glutamine. It should be noted that *S. cerevisiae* has *snz* homologues, but not *pdxA/J* homologues like *B. subtilis*. According to above two grouping, *B. subtilis* and *S. cerevisiae* belong to the same group, but *E. coli* does not.

These two kinds of grouping are still uncertain. However, combining the results of this study and the groupings, it is likely that there are at least two different B_6 biosynthetic pathways among microorganisms. It is also likely that the pathways of *E. coli* and *B. subtilis* are different.

At present, there is no reliable precursor or biosynthetic pathway of B_6 in *B. subtilis*. Therefore, it is difficult to discuss precursors or the pathway of *B. subtilis*. In this study, the author found genes, which could be involved in B_6 biosynthesis in *B. subtilis*. Elucidation of the roles of YaaD and YaaE will provide a clue to B_6 biosynthesis of *B. subtilis*.

GENERAL SUMMARY

In Chapter I, glycolaldehyde (GA)-forming route in relation to vitamin B_6 (B_6) biosynthesis was investigated. GA is a precursor, and formed by GA dehydogenase (GADH) in *Escherichia coli.* It was found that in *Bacillus subtilis,* GADH did not have GA-forming activity. Involvement of another possible GA-forming enzyme, glyoxal reductase (GR) was also examined. GR showed GA-forming activity, but did not affect B_6 biosynthesis. As a result, it was indicated that neither GADH nor GR is involved in B_6 biosynthesis in *B. subtilis.* It was considered that the GA-forming route of *B. subtilis* is different from the route of *E. coli*, and GA is not a precursor of B_6 in *B. subtilis.*

In Chapter II and III, involvement of *serC* and *tkt* was investigated. In *E. coli, serC* and *tkt* are considered to be involved in synthesis of a proposed precursor of B_6, 4-hydroxy-L-threonine. *serC* and *tkt* were disrupted in *B. subtilis.* Both disruptants did not require B_6, and retained the ability to synthesize B_6. Therefore, it was unlikely that *serC* and *tkt* are involved in B_6 biosynthesis in *B. subtilis.*

In Chapter IV, *yqiE* and *yluB* were investigated. In *E. coli,* homologues of *yqiE* and *yluB* are considered to be involved in synthesis and metabolism of another proposed precursor of B_6, 1-deoxy-D-xylulose. Conditional mutants of *yqiE* and *yluB* were constructed. Both mutants synthesized the same amount of B_6 as wild type with or without induction of transcription. Alternation of transcriptional levels of *yqiE* and *yluB* did not affect B_6 production, and therefore, *yqiE* and *yluB* are considered to be not involved in B_6 biosynthesis in *B. subtilis.*

In Chapter V, involvement of *yaaD* and *yaaE* was examined. *yaaD* is a homologue of genes, which are considered to be involved in B_6 biosynthesis in *Cercospora nicotianae*

and *Aspergillus nidulans*. *yaaE* is the downstream gene of *yaaD*. Disruptants of *yaaD* and *yaaE* required B_6. Transcriptions of *yaaD* and *yaaE* were not repressed by either pyridoxine or pyridoxal. However, the result indicated that *yaaD* and *yaaE* could be involved in B_6 biosynthesis in *B. subtilis*.

This thesis demonstrated that genes involved in B_6 biosynthesis in *E. coli* are not involved in that in *B. subtilis*. It was also shown that *yaaD* and *yaaE* are involved in B_6 biosynthesis in *B. subtilis*. The results showed that B_6 biosynthetic pathway of *B. subtilis* is different from the pathway of *E. coli*. *yaaD* and *yaaE* are the first genes identified as the genes involved in B_6 biosynthesis in *B. subtilis*. The roles of YaaD and YaaE in B_6 biosynthesis are currently unknown. Elucidation of the functions of YaaD and YaaE will provide a clue to B_6 biosynthetic pathway of *B. subtilis*.

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