Chapter I

Production of vitamin B_6 in *Rhizobium*

Introduction

In the past several decades, screening for vitamin B_6 overproducers has been done extensively and has yielded the following microorganisms (Table 1): a *Klebsiella* strain producing 2 mg of pyridoxamine per liter of culture,[21] *Achromobacter cycloclastes* A.M.S. 6201 producing 3 to 4 mg of vitamin B_6 per liter of culture,[22] marine organisms, a *Vibrio* strain M-31 and a *Flavobacterium* strain 238-7 respectively yielding 5 mg and 18 mg of vitamin B_6 per liter of culture broth,[23] mutants of a *Bacillus* strain excreting 2 to 5 mg of vitamin B_6 per liter of growth medium,[24] a mutant of *Saccharomyces microsporus* producing 3 mg of vitamin B_6 per liter of culture medium,[25] a mutant of *Saccharomyces marxianus* excreting about 2 mg of vitamin B_6 per liter of growth medium,[26] and *Pichia guilliermondii* Wickerham NK-2 producing approximately 25 mg of vitamin B_6 per liter of culture broth.[27] But no promising industrially applicable strain has yet been obtained.

Table 1. Production of Vitamin B_6 by Microorganisms

Microorganisms	Vitamin B_6 (mg/l)	References
Klebsiella sp.	2	Suzue *et al.*[21]
Achr. cycloclastes A.M.S. 6201	3~4	Ishida *et al.*[22]
Vibrio sp. M-31	5	Tani *et al.*[23]
Flavobacterium sp. 238-7	18	Tani *et al.*[23]
B. subtilis mutants	2~5	Pflug *et al.*[24]
S. microsporus mutant	3	Scherr *et al.*[25]
S. marxianus mutant	2	Pardini *et al.*[26]
Pichia guilliermondii NK-2	25	Nishino *et al.*[27]

Therefore, I did a microbial screening search for vitamin B_6 overproducers. This chapter describes the microbial screening for vitamin B_6 producers, the taxonomy of a vitamin B_6 overproducing strain, and the production of vitamin B_6 in *Rhizobium* strains purchased from the culture collection.

Materials and Methods

Chemicals. The standard mixture of four nucleotides and nuclease P_1 were purchased from Yamasa Shoyu Co., Ltd., (Choshi, Chiba, Japan) and the standard solution containing 22 kinds of fatty acid ester and BCl_3-methanol for esterification of fatty acid were from Supelco, Inc. (Bellefonte, PA., USA). Yeast extract and a Gram Stain Set were from Difco Laboratories (Detroit, MI., USA), bouillon was from Kyokutoh Pharmaceutical Co., Ltd., Tokyo, and Polypepton, Polypepton-S, Polypepton-P1, and Polypepton-Y were from Nihon Pharmaceutical Co., Ltd., Tokyo, Japan. Pyridoxine Assay Medium for biological measurement of vitamin B_6 was from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. Vitamin B_6 compounds, pyridoxol, pyridoxal, pyridoxamine, pyridoxal phosphate, pyridoxamine phosphate, and 4′-deoxypyridoxol, were from Sigma Chemical Co., Ltd., (St. Louis, MO., USA).

Microorganisms. About 1,590 bacteria isolated from soil were used for screening for vitamin B_6 producers. Thirty-three *Rhizobium* strains used for testing productivity of vitamin B_6 were obtained from IFO and IAM. *Saccharomyces carlsbergensis* ATCC 9080 was used to measure vitamin B_6.

Media. The medium for the isolation of bacteria from soil was composed of 1% bouillon, 0.2% yeast extract, and 1.5% agar (pH 7.0) designated as BY agar. The medium for agar culture of bacterial isolates was composed of 2% glucose, 2% Polypepton, 0.2% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 5H_2O$, and 1.5% agar (pH 6.8). A seed medium for flask fermentation of *Rhizobium* strains was composed of 1% glucose, 0.5% Polypepton, 0.2% yeast extract, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 5H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$ (pH 6.8). The production medium for the flask fermentation of vitamin B_6 was composed of 4% glucose, 2% Polypepton, 0.2% yeast extract, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% $MnSO_4 \cdot 5H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$ (pH 6.8).

Isolation of microorganisms from soil. One gram of soil sample was suspended in 5 ml of 0.85% sterile saline, and serial 10-fold dilutions were made. Then 0.1 ml of each serial diluent was spread over BY agar containing 10 μg/ml of cycloheximide. Colonies grown at 28°C were transferred onto fresh BY agar and used for screening for vitamin B_6 producers.

Guanine-plus-cytosine content of the DNA.

DNA of an isolate, 28-21, was extracted and purified by the method of Saitoh and Miura.[28] After the DNA was hydrolyzed to mononucleotides with nuclease P_1, the guanine-plus-cytosine (GC) content of the DNA was measured by high-pressure liquid chromatography (HPLC).[29]

Cellular fatty acid composition. The cellular fatty acid composition of isolate 28-21 was analyzed by the method of Shaw.[30] Lyophilized cells were stirred in 15% KOH methanolic solution at 70°C for 4 h, and then fatty acids were extracted with ethyl ether under acidic conditions with H_2SO_4 (pH 2.0). After esterification with BCl_3-methanol, gas chromatographic analysis of the fatty acid methyl esters was done using a WSCOT SE30 capillary column (0.25 mm × 25 m).

Cultivation and growth. One loopful of cells was inoculated into tubes containing 10 ml of seed medium, and then the tubes were shaken for 16 h on a reciprocal shaker (285 rpm) at 28°C. For flask fermentation, 4 milliliters of the seed culture was inoculated into a 500-ml flask containing 200 ml of medium, and the flask was shaken on a rotary shaker (180 rpm) at 28°C. Cell growth was estimated turbidometrically at 600 nm.

Measurement of vitamin B_6. The content of vitamin B_6 was measured by its turbidity using *S. carlsbergensis* ATCC 9080[31] with the following procedure. The supernatant of culture broth and a standard solution of pyridoxol were diluted in distilled water. Two hundred microliters of the diluted solution, 1.5 ml of distilled water and 40 μl of 1.155 N H_2SO_4 were added to tubes in this order. These tubes were autoclaved at 121°C for 20 min. After cooling, 1.5 ml of Pyridoxine Assay Medium with *S. carlsbergensis* ATCC 9080 was added to the tubes (final OD_{600}=0.028), which were placed at an incline of 30° and incubated without shaking at 28°C for 17 h. The cell growth was stopped by the addition of 5 ml of 0.2 N HCl, and the absorbance of the sample was measured at 660 nm. The amount of vitamin B_6 in a sample was measured by comparing the turbidity of the sample with those with the standard solutions of pyridoxol.

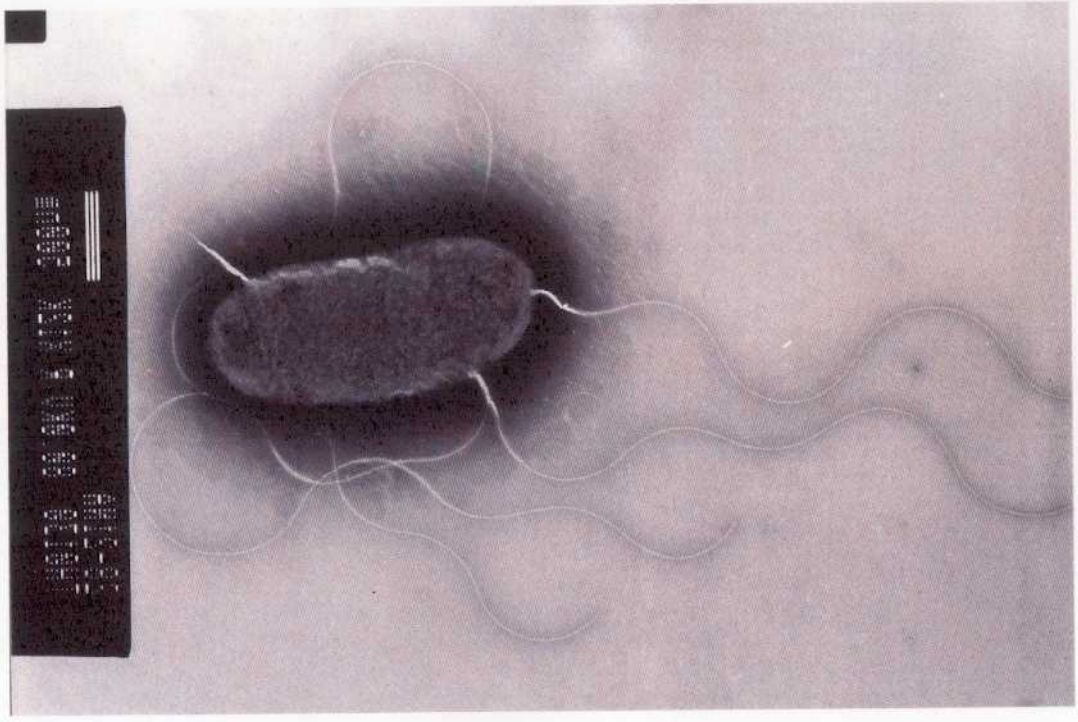
HPLC analysis of vitamin B_6. HPLC analysis of vitamin B_6 was done by the internal standard method with 4′-deoxypyridoxol. The analytical conditions were as follows: column, Capcell pak C18 SG120 (4.6 × 250 mm) (Shiseido Co., Ltd., Tokyo, Japan); mobile phase, 0.1 M sodium perchlorate, 0.1 M potassium phosphate and 2% acetonitrile (pH 3.5); column temperature, 25–26°C; flow rate, 1.0 ml/min; and detector, ultraviolet (UV) (at 292 nm). To prepare the samples for HPLC, 100 microliters of the solution containing 100 mg/l of 4′-deoxypyridoxol (internal substance) was added to 400 μl of the standard solutions of pyridoxol and the supernatant from the culture broth, and then the mixture was put on the HPLC column.

Results and Discussion

Screening of vitamin B_6 producers I screened soil isolates for vitamin B_6 producers. I used a rapid and convenient method to isolate vitamin B_6 producers, which is very critical for the primary selection stage. Bacteria were isolated from 49 soil samples by the method described in Materials and Methods. About 1,590 isolates were inoculated onto agar blocks of the medium for vitamin B_6 production and incubated for 2 d at 28°C. The plates were irradiated with UV light for 2 h to sterilize the cells, and the vitamin B_6 produced was microbiologically detected on Pyridoxine Assay Medium agar with a vitamin B_6 indicator strain, *S. carlsbergensis* ATCC 9080. The diameters of growth halos, which depended on the amount of vitamin B_6, were measured. Isolates producing halos larger than 30 mm in diameter amounted to 4.4% (70 strains) of the isolates, while about 72% (1,143 strains) of the isolates produced halos less than 25 mm, indicating the production of only a small amount of vitamin B_6. Among the high producers of vitamin B_6, strain 28-21 was selected as the best producer.

Taxonomy of vitamin-B_6-overproducing organism

Strain 28-21 was a Gram-negative aerobe having the form of motile rods with three to five peritrichous flagella, and never produced spores (Electron Microphotograph). As the taxonomic characteristics are summarized in Table 2, strain 28-21 belongs to the family *Rhizobiaceae* which is classified into four genera, *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, and *Phyllobacterium*.



Electron Microphotograph of Strain 28-21

Table 2. Taxonomic Characteristics of Strain 28-21

Characteristics	Strain 28-21
Gram staining	Negative
Cell shape	Rod
Cell size	0.7–0.9 × 1.5–3.8 μm
Motility	Motile
Flagellar arrangement	Peritrichous
Spore formation	Negative
Growth-behavior to O_2	Aerobic
Assimilation of C_1 compound	Negative
Growth on or in peptone	Positive
Formation of acetic acid from ethanol	Negative
Tolerance of NaCl	< 3.0%
Oxidase	Positive
Catalase	Weakly positive
Isoprenoid quinone	Q-10
G+C mol% of the DNA	60.7
Colony size of 1–2 mm in diameter	Yes
Formation of star-shaped clusters	Negative

Recently, however, one new genus *Azorhizobium* was reported by Dreyfus *et al.*[32] Strain 28-21 was compared with type strains of those genera with respect to the three characteristics of GC content, colony sizes within 3 d on yeast extract-mannitol-mineral salts agar, and formation of star-shaped clusters in carrot juice medium. The results indicated that strain 28-21 was differentiated from the genera *Azorhizobium*, *Bradyrhizobium*, and *Phyllobacterium*, and that it belonged to the genus *Rhizobium* or *Agrobacterium*. *Rhizobium* and *Agrobacterium* are known to show closely related taxonomic characteristics and close DNA-DNA and rRNA-DNA similarities. Yokota and Sakane[33] reported that the species of *Rhizobiaceae* could be easily distinguished on the basis of cellular fatty acid profiles, and that the method was useful for the identification of the bacteria. The cellular fatty acid profile of strain 28-21 was analyzed by gas chromatography. From the hydroxy fatty acid profile, strain 28-21 had 3-OH 16:0, 3-OH 18:0, and 3-OH *anteiso*-15:0 (Table 3). This profile coincides with that of *R. leguminosarum*. Therefore, strain 28-21 was decided to belong to *R. leguminosarum* and was designated as *R. leguminosarum* strain 28-21.

Table 3. Comparison of Cellular Hydroxy Fatty Acid Profiles Between Strain 28-21 and *Rhizobiaceae*

Microorganisms	Cellular hydroxy fatty acid					
	2-hydroxy acids	3-hydroxy acids				
		12:0	*iso*-13:0	*anteiso*-15:0	16:0	18:0
strain 28-21 (*Rhizobium*)	−	−	−	+	+	+
R. leguminosarum	−	−	−	+	+	+
R. meliloti	−	−	−	−	±	+
R. fredii	−	−	−	−	±	+
R. galegae	−	−	−	−	+	+
R. loti (*Agrobacterium*)	−	+	+	−	+	+
Agrobacterium biovar 1	−	−	−	−	+	−
Agrobacterium biovar 2	+	−	−	+	+	+
Agrobacterium biovar 3	+	−	−	−	+	+
Agrobacterium rubi	−	−	−	−	±	−

All the data except for strain 28-21 are from Yokota and Sakane.[33]

Vitamin B_6 production by Rhizobium strains

The production of vitamin B_6 by *R. leguminosarum* strain 28-21 was examined together with 33 *Rhizobium* strains obtained from the culture collections. The flask fermentation was done for 7 d in production medium (but 2% glucose and 1% Polypepton as carbon and nitrogen sources for *R. leguminosarum*). The culture broth was centrifuged, and vitamin B_6 content in the supernatant was measured. Large amounts of vitamin B_6 were produced in many strains, such as *R. meliloti* IFO 14782, *R. huakii* IFO 15243, and *R. loti* IFO 14998 as well as *R. leguminosarum* strain 28-21 (Table 4). The best producer was *R. meliloti* IFO 14782, which produced 51 mg of vitamin B_6 per liter. The productivity was 2.2 times higher than that of *R. leguminosarum* strain 28-21.

Table 4. Productivity of Vitamin B_6 in *Rhizobium* Strains

Rhizobium strains	Vitamin B_6 (mg/l)
R. meliloti IFO 14782	51
R. meliloti IAM 12035	3
R. huakii IFO 15243	20
R. huakii IFO 15244	0
R. leguminosarum strain 28-21	23
R. leguminosarum IFO 13337	0
R. leguminosarum IFO 13338	0
R. leguminosarum IFO 14168	5
R. leguminosarum IFO 14778	10
R. leguminosarum IFO 14784	2
R. leguminosarum IFO 14785	0
R. leguminosarum IFO 14994	2
R. leguminosarum IFO 14995	3
R. tropici IFO 15247	9
R. galegae IFO 14965	8
R. loti IFO 13336	1
R. loti IFO 14779	1
R. loti IFO 14996	1
R. loti IFO 14997	0
R. loti IFO 14998	10
R. loti IFO 14999	0
R. loti IFO 15000	4
R. loti IAM 13630	1
R. loti IAM 13633	0
R. loti IAM 13634	2
R. fredii IFO 14780	3
R. fredii IAM 13626	0
R. fredii IAM 13627	0
Rhizobium sp. IAM 13623	6
Rhizobium sp. IAM 13628	0
Rhizobium sp. IAM 13629	0
Rhizobium sp. IAM 13631	5
Rhizobium sp. IAM 13632	0
Rhizobium sp. IAM 13635	3

Optimization for vitamin B_6 production

A media study for vitamin B_6 production by *R. meliloti* IFO 14782 was done. I examined the effects of peptone at the concentration of 2 or 4% on production of vitamin B_6. As the result, the medium with 4% Polypepton was better than that with 2% for production of vitamin B_6, and the medium with 4% Polypepton-S (a pancreatic digest of soybean-casein) gave the best value of vitamin B_6 (60 mg/l) (Table 5). A pancreatic digest of vegetable casein might be more desirable for production of vitamin B_6 than that of animal ones such as Polypepton, Polypepton-P1, and Polypepton-Y.

Table 5. Effects of Peptone on Productivity of Vitamin B_6 in *R. meliloti* IFO 14782

Peptones	(%)	Cell growth (OD_{600})			Vitamin B_6 (mg/l)		
		2 d	3 d	6 d	2 d	3 d	6 d
Polypepton	2	15	23	27	7.7	21	34
	4	10	22	29	5.2	19	43
Polypepton-S	4	24	33	31	13	30	60
Polypepton-P1	4	16	25	31	8.4	23	48
Polypepton-Y	4	13	19	26	7.6	20	40

Then, the additional effect of yeast extract on production of vitamin B_6 was examined in production medium with 4% Polypepton-S as nitrogen source. When the medium was supplemented with 0.8% yeast extract, production of vitamin B_6 showed the highest value of 84 mg per liter in 7 d, and about 90% of vitamin B_6 in the culture broth was pyridoxol (Table 6).

Table 6. Effects of Yeast Extract on Production of Vitamin B_6 in *R. meliloti* IFO 14782

Yeast extract (%)	Cell growth (OD_{600})			Vitamin B_6 (mg/l)			Pyridoxol (mg/l)		
	3 d	5 d	7 d	3 d	5 d	7 d	3 d	5 d	7 d
0.2	37	36	37	29	68	80	26	56	64
0.4	38	37	40	28	69	79	26	57	73
0.6	41	41	45	30	58	83	20	59	76
0.8	38	40	46	28	60	84	20	58	79

Vitamin B_6 in the supernatant was also analyzed by HPLC (Fig. 1), and furthermore, each fraction eluted from HPLC was assayed by turbidity method with *S. carlsbergensis* ATCC 9080. The fractions corresponding to the retention times for pyridoxol and pyridoxamine in HPLC analysis were confirmed to support the growth of *S. carlsbergensis* ATCC 9080, but an unknown peak with a retention time of 4.6 min did not support the growth of *S. carlsbergensis* ATCC 9080 (data not shown).

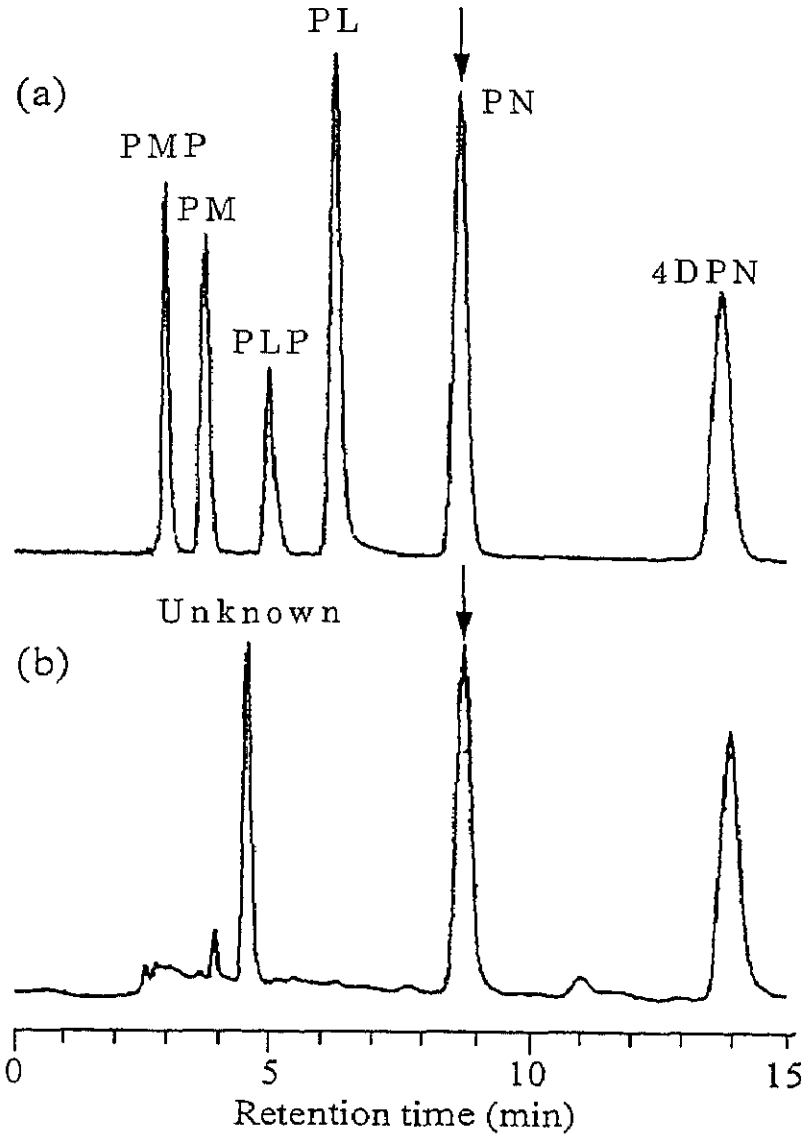


Fig. 1. High Pressure Liquid Chromatography Profile of Vitamin B_6 in Supernatant of *R. meliloti* IFO 14782.

The peak of pyridoxol is indicated by an arrow. (a) Standard solution of vitamin B_6: Six peaks are pyridoxamine phosphate (PMP), pyridoxamine (PM), pyridoxal phosphate (PLP), pyridoxal (PL), pyridoxol (PN), and 4′-deoxypyridoxol (4DPN) from left. (b) Supernatant of 7-d culture broth.

Through our screening search for vitamin B_6 overproducers, I obtained one bacterium, which belongs to the genus *Rhizobium*. Based on this finding, I surveyed various *Rhizobium* strains available from the culture collections and found that all of them are, in general, vitamin B_6 overproducers. There have been no reports dealing with such overproduction of vitamin B_6. It must be very interesting to clarify the reason why *Rhizobium* strains are capable of producing high amounts of vitamin B_6. In this study, I selected *Rhizobium meliloti* IFO 14782 as the best vitamin B_6 producer among all the *Rhizobium* strains I tested. This strain would be a good starting material for further improvement of its vitamin productivity by classical mutagenesis as well as genetic engineering in the future.