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Regioselective Glucosylation of Pyridoxine by Microorganisms

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Microorganisms from culture collections and isolates from nature were screened for the ability to catalyze the regioselective glucosylation of pyridoxine (PN) to produce pyridoxine 5′-α-D-glucoside (PN-5′-α-G) or pyridoxine 4′-α-D-glucoside (PN-4′-α-G). Trans-glucosylation activity specific to 5′-position of PN was found in fungi belonging to genera such as *Coriolus* and *Verticillium*, and activity at the 4′-position of PN was found in bacteria belonging to genera such as *Bacillus* and *Serratia*. From 100 mM PN, intact cells of *Verticillium dahliae* TPU 4900 produced 42 mM (13.9 mg/mL) PN-5′-α-G after 70 h of reaction. Intact cells of *Bacillus cereus* TPU 5504 produced 33 mM (10.9 mg/mL) PN-4′-α-G after 19 h of reaction. The selectivities for 5′- and 4′-positions were 80% and 90%, respectively.

Key words: *Verticillium*; *Coriolus*; *Bacillus*; pyridoxine 5′-α-D-glucoside; pyridoxine 4′-α-D-glucoside

Pyridoxine α-D-glucoside (PN-α-G) is nutritionally important as vitamin B_6,[1] and is more stable than PN against light and heat.[2] Two compounds, pyridoxine 5′-α-D-glucoside (PN-5′-α-G) and pyridoxine 4′-α-D-glucoside (PN-4′-α-G) are positional isomers of PN-α-G[3] (Fig. 1), and the former is hydrolyzed by liver cells to PN more easily than the latter.[4] Anomer selective and regioselective introduction of the glucosyl group into PN is needed for the production of PN-5′-α-G. In the chemical glucosylation of PN in the 5′- or 4′-position, it is necessary to protect the functional groups of PN and glucose, and to quench the β-anomer with β-glucosidase.[3]

The enzymatic glucosylation of PN with several microorganisms and enzymes has been studied, but selectivity for the 5′-position and yield of PN-α-G are low. *Micrococcus luteus* (formerly *Sarcina lutea*) produces PN-α-G that consists of a large amount of PN-5′-α-G and a small amount of PN-4′-α-G (molar ratio, about 4:1) from sucrose and PN.[3,5] Furthermore, α-glucosidase from *Mucor javanicus* forms PN-5′-α-G and PN-4′-α-G at the molar ratio of 1:1 from dextrin and PN.[6] In addition, cyclomaltodextrin glucanotransferase (CGTase) from *Bacillus stearothermophilus* and *Paenibacillus macerans* (formerly *Bacillus macerans*) formed PN-5′-α-G and PN-4′-α-G at the molar ratio 1:1.4 to 1:1.8 from dextrin and PN.[6,7] 4′-Position-selective α-glucosidases from baker's yeast, *B. stearothermophilus*, and

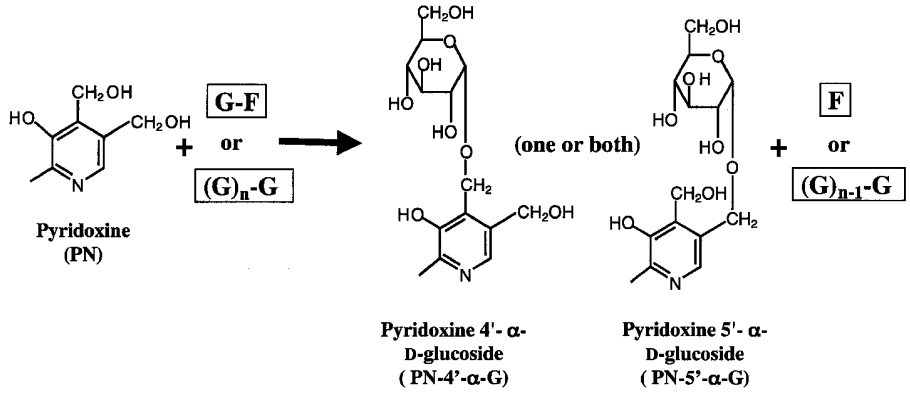

Fig. 1. Enzymatic Synthesis of Pyridoxine α-D-Glucoside.
Abbreviations: G–F, sucrose; $(G)_n$-G, dextrin; F, fructose.

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Abbreviations: CGTase, cyclomaltodextrin glucanotransferase; PN, pyridoxine; PN-5′-α-G, pyridoxine 5′-α-D-glucoside; PN-4′-α-G, pyridoxine 4′-α-D-glucoside; PN·HCl, pyridoxine hydrochloride

rice produce only PN-4′-α-G from maltose and PN.[8] There has been no report about a microorganism or enzyme that transfers a glucosyl group to the 5′-position of PN in high yield.

To produce PN-5′-α-G as a useful and stable vitamin B_6 derivative with easy isolation and purification, it is necessary to obtain microorganisms that have the activity of transglucosylation specific to 5′-position of PN. In this paper, we describe the results of screening for microorganisms catalyzing regioselective formation of PN-α-G from sucrose, dextrin, or both.

Materials and Methods

Chemicals. Pyridoxine hydrochloride (PN·HCl) was provided to us by Daiichi Fine Chemical Co., Ltd. (Toyama, Japan). Dextrin (TK-16) was purchased from Matsutani Chemical Industries Co., Ltd. (Hyogo, Japan). CGTase (from *Paenibacillus macerans*) was purchased from Amano Enzyme Inc. (Aichi, Japan). Glucoamylase (from *Rhizopus niveus*) was purchased from Seikagaku Co. (Tokyo, Japan). All other chemicals used were commercially available and were of analytical grade.

Medium. Medium I for screening contained 2.0% dextrin, 2.0% sucrose, 1.0% peptone, 0.05% yeast extract, 0.5% K_2HPO_4, 0.1% KH_2PO_4, 0.02% $FeSO_4 \cdot 7H_2O$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $MnSO_4 \cdot 5H_2O$, and 0.1% PN·HCl in tap water at pH 7.0. The medium for plate was solidified by the addition of 2.0% agar. Medium II for flask culture of microorganisms forming PN-5′-α-G contained 4% soluble starch, 1% Esusan meat (Ajinomoto Co., Inc., Tokyo), 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, and 0.1% PN·HCl in tap water at pH 7.0.

Microorganisms. Microorganisms from culture collections (907 strains), isolates from waste water of industrial plants (Daiichi Fine Chemical Co., Ltd.) of PN·HCl (53 strains), and isolates from contaminated PN-α-G solutions (10 strains) were screened. Type cultures were from the Institute of Molecular and Cellular Biosciences (IAM), University of Tokyo; the Institute of Fermentation (IFO), Osaka, Japan; the Japan Collection of Microorganisms (JCM), Tokyo; and our laboratory (TPU, Toyama Prefectural University).

We suspended 1 mL of waste water in 15 mL of medium I (final concentration of PN·HCl, 5%) in a 100-mL flask, and the flask was shaken for 7–10 d at 30°C. One milliliter of the culture was used to inoculate freshly prepared medium, which was incubated. This procedure was repeated once more, and a small volume of the culture medium was spread on plates of medium I. Separately, a mass of microorganisms that had grown spontaneously on five solutions of PN-α-G was suspended in a small volume of sterilized 0.1% Tween 80 and spread on plates of medium I. The plates were incubated at 30°C and colonies were isolated.

Screening. Microorganisms from culture collections and isolates from nature were grown on 5 mL of medium I in test tubes ($\phi 16.5 \times 165$ mm) for 2–14 d (until the microorganisms were growing well) at 30°C with shaking, and some fungal strains were cultured at 25°C. The culture broth was boiled for 10 min, and centrifuged ($16{,}000 \times g$, for 10 min) to remove the cells, the supernatant was examined by HPLC.

Preparation of authentic samples of PN-5′-α-G and PN-4′-α-G. The method of Suzuki *et al.*[6] was modified for preparation of authentic samples of PN-5′-α-G and PN-4′-α-G with the use of CGTase. The reaction mixture consisted of 12 mL of 100 mM potassium phosphate buffer (pH 7.0) containing 0.37 g (1.8 mmol) of PN·HCl, 0.96 g of dextrin, 0.10 mg $CaCl_2 \cdot 2H_2O$, and 1440 units of CGTase (one unit of CGTase activity was defined by the method of Tilden and Hudson[9]). The reaction was done at 30°C for 144 h in the dark and was stopped by boiling of the mixture for 10 min. Glucoamylase treatment (30°C, 17 h) was done to convert pyridoxine α-maltosides, that appeared as by-products, to PN-5′-α-G or PN-4′-α-G. After the reaction mixture was evaporated under reduced pressure and put on a Sephadex LH-20 column ($\phi 14.5 \times 1200$ mm, Amersham Bioscience Corp., Piscataway, NJ), a mixture of PN-5′-α-G and PN-4′-α-G was eluted with 20% (v/v) methanol. The mixture was injected into an ODS column for preparative HPLC (YMC-pack ODS column SH-343-5, $\phi 20 \times 250$ mm, YMC Co., Ltd., Kyoto) and then eluted with 4% (v/v) methanol at the flow rate of 3.5 mL/min. The fractions containing only PN-5′-α-G were combined and evaporated to dryness, and 94 mg of powder was obtained. From the fractions containing only PN-4′-α-G, 74 mg of powder was obtained.

Analysis of PN-α-G. PN, PN-5′-α-G, and PN-4′-α-G were analyzed by HPLC with a Cosmosil $5C_{18}$AR column ($\phi 4.6 \times 150$ mm, Nakalai Tesque, Inc., Kyoto) with detection at 325 nm. The mobile phase was 1% (v/v) methanol, and the flow rate was 0.8 mL/min at 30°C. Selectivity (%) for the 5′-position of PN was calculated as the amount of PN-5′-α-G divided by the amounts of sum of PN-5′-α-G and PN-4′-α-G, with the result multiplied by 100. The selectivity at the 4′-position was calculated in a similar way.

Synthesis of PN-5′-α-G by intact cells. Coriolus

Table 1. Number of Strains with High Regioselectivity

			Number of microorganisms tested		Number of strains with 5′-position selectivity*			Number of strains with 4′-position selectivity*		
			Genera	Strains	Yield of PN-5′-α-G in culture, mM 0.08–0.75	0.76–1.5	>1.5	Yield of PN-4′-α-G in culture, mM 0.08–0.75	0.76–1.5	>1.5
Culture collection		Bacteria	37	307	4 (0)	1 (0)	0 (0)	20 (9)	10 (5)	0 (0)
		Actinomycetes	8	87	1 (0)	0 (0)	0 (0)	2 (0)	1 (0)	0 (0)
		Yeasts	23	235	4 (1)	0 (0)	0 (0)	6 (1)	1 (1)	0 (0)
		Fungi	61	278	36 (12)	14 (12)	6 (5)	12 (0)	5 (0)	1 (0)
		Total	129	907	45 (13)	15 (12)	6 (5)	40 (10)	17 (6)	1 (0)
Isolated strain	from waste water	Not fungi		40	0 (0)	0 (0)	0 (0)	1 (0)	1 (1)	0 (0)
		Fungi		13	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	from contaminated PN-α-G-solution	Not fungi		6	0 (0)	0 (0)	0 (0)	2 (0)	0 (0)	0 (0)
		Fungi		4	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
		Total		63	0 (0)	1 (0)	0 (0)	3 (0)	1 (1)	0 (0)

* Regioselectivity was calculated at the amount of PN-5′-α-G or PN-4′-α-G divided by the amounts of sum of PN-5′-α-G and PN-4′-α-G, with the result multiplied by 100. Strain with regioselectivity of more than 75% was defined as a strain with 5′- or 4′-position selectivity.
The numbers of strains with regioselectivity of more than 90% are given parentheses.

fibula IFO 4949 and *Coriolus pubescens* IFO 9782 were cultured on 100 mL of medium II in 500-mL flasks on a rotary shaker (200 rpm) at 25°C for 7 d. *Verticillium dahliae* TPU 4900 was cultured on the same medium at 20°C for 11 d. The cells were harvested by filtration with suction and then washed with distilled water. The reaction mixture for PN-5′-α-G synthesis consisted of 1.2 mL of 100 mM potassium phosphate buffer containing 24.7 mg (0.12 mmol) of PN·HCl, 24 mg of dextrin, and the cells harvested from 1.0 mL of culture broth (52 mg of *C. fibula* IFO 4949, 50 mg of *C. pubescens* IFO 9782, or 109 mg of *V. dahliae* TPU 4900, wet weights). The reactions were done at pH 5.0–6.5 (adjusted with NaOH) and 40°C for 96 h with shaking in the dark, and then 84 mg of dextrin was added each of three times, at 20, 40, and 70 h, and an equal amount of cells was added at 40 h.

Synthesis of PN-4′-α-G by intact cells. *Bacillus cereus* TPU 5504 was cultured on 150 mL of medium I in a 500-mL flask on the rotary shaker at 30°C for 4 d. The cells were harvested by centrifugation (8200 $\times g$ for 15 min) and then washed with distilled water. The reaction mixture for PN-4′-α-G synthesis consisted of 250 mL of 100 mM potassium phosphate buffer containing 5.1 g (25 mmol) of PN·HCl, 5.0 g of dextrin, and the cells harvested from 500 mL of culture broth (9.7 g, wet weight) in a 500 mL-flask. The reactions were done at pH 6.0–7.5 (adjusted with NaOH) and 40°C for 48 h with stirring in the dark. An equal amount of dextrin was added at 6 h and 26 h, and an equal amount of cells was added at 26 h.

Results

Screening for microorganisms forming PN-α-G from stock cultures

Nine hundred and seven strains from stock cultures of TPU, IAM, IFO, and JCM were grown in medium I and then analyzed. Strains that yielded PN-5′-α-G in more than 1.5% molar yield (about 0.08 mM) and had 5′-position selectivity in more than 75% were selected as 5′-position-selective strains, and 4′-position-selective strains were selected in a similar way. The numbers of selective strains of both kinds are listed in Table 1.

Of the bacteria examined (307 strains in 37 genera), there were more 4′-position-selective strains than that of 5′-position selective ones. 4′-Position selectivity was found in nine genera: *Arthrobacter*, *Bacillus*, *Corynebacterium*, *Edwardsiella*, *Hafnia*, *Ochrobactrum*, *Salmonella*, *Serratia*, and *Xanthobacter* (31 strains, 10% of tested bacteria). 5′-Position-selectivity was found in the genera *Corynebacterium* and *Paenibacillus* (4 strains). Nonselective activity to form PN-α-G was seen in five genera of bacteria: *Cedecea*, *Enterobacter*, *Kocuria*, *Micrococcus*, and *Pseudomonas*. Neither PN-5′-α-G nor PN-4′-α-G was produced in more than 1.5% molar yield by the strain of *Achromobacter*, *Acinetobacter*, *Aeromonas*, *Agrobacterium*, *Alcaligenes*, *Citrobacter*, *Comamonas*, *Curtobacterium*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Klebsiella*, *Kluyvera*, *Methylobacterium*, *Morganella*, *Pantoea*, *Pimelobacter*, *Proteus*, *Providencia*, *Thiobacillus*, *Variovorax*, or *Yersinia*. Of bacteria that accumulated PN-5′-α-G or PN-4′-α-G in more than 15% molar yield (0.75 mM) in the culture broth, there were ten 4′-position-selective strains (5 strains with selectivity of more than 90%), but *Paenibacillus alvei* IFO 3343 was the only 5′-position-selective strain.

Of the actinomycetes (87 strains in 8 genera), *Pseudonocardia autotrophica* TPU 3870 was the only 5′-position-selective strain, but three strains of the genus *Nocardia* or *Rhodococcus* were 4′-position-selective. The actinomycetes from *Gordona*, *Mycobacterium*, and *Streptomyces* had nonselective

PN-α-G-forming activity. Strains from *Actinomyces*, *Nocardioides*, and *Rothia* formed neither PN-5′-α-G nor PN-4′-α-G in more than 1.5% molar yield.

Of yeasts (235 strains in 23 genera), three strains of *Cryptococcus* were 5′-position-selective, and six strains from *Nakazawaea*, *Saccharomyces*, and *Zygosaccharomyces* were 4′-position-selective. Only one strain, *Saccharomyces heterogenicus* TPU 1043, both produced PN-4′-α-G with a high molar yield (27%) in the culture broth and had high 4′-position selectivity (97%). Yeasts with nonselective PN-α-G-forming activity were found in seven genera: *Candida*, *Debaryomyces*, *Hansenula*, *Pichia*, *Rhodotorula*, *Schizosaccharomyces*, and *Sporobolomyces*. Strains belonging to 12 genera, *Brettanomyces*, *Geotrichum*, *Hanseniaspora*, *Kloeckera*, *Kluyueromyces*, *Lipomyces*, *Rhodosporidium*, *Saccharomycodes*, *Saccharomycopsis*, *Torulopsis*, *Trichosporon*, and *Williposis*, yielded neither PN-5′-α-G nor PN-4′-α-G in more than 1.5% molar yield.

Of the 278 strains in 61 genera of fungi tested, 56 strains from *Coriolus*, *Eurotium*, *Flammulina*, *Ganoderma*, *Gliocladium*, *Helicostylum*, *Mortierella*, *Pithomyces*, *Schizophyllum*, *Trametes*, and *Verticillium* had 5′-position selectivity. Seventeen of them (30% of the 5′-position selective fungi) produced PN-5′-α-G in more than 15% molar yield (0.75 mM) and with 5′-position selectivity of more than 90%. Seventeen other strains (6% of the fungi tested) produced PN-4′-α-G in high 4′-position selectivity, but all at less than 90%. These strains were in six genera: *Beauveria*, *Fusarium*, *Mortierella*, *Penicillium*, *Tritirachium*, and *Verticillium*. Fungi forming PN-α-G nonregioselectively were found among *Acremonium*, *Armillariella*, *Aspergillus*, *Caldariomyces*, *Chaetosartorya*, *Cladosporium*, *Dactylaria*, *Edyuillia*, *Gibberella*, *Neurospora*, *Panus*, and *Phytophthora*. Strains from *Absidia*, *Arthroderma*, *Aureobasidium*, *Chaetomium*, *Chrysosporium*, *Coprinus*, *Cryptoporus*, *Cunninghamella*, *Cylindrocarpon*, *Daedalea*, *Daedaleopsis*, *Exophiala*, *Gloeophyllum*, *Grifola*, *Hypocrea*, *Irpex*, *Keratinomyces*, *Laetiporus*, *Lentinus*, *Lepista*, *Mucor*, *Neosartorya*, *Neurospora*, *Pholiota*, *Phycomyces*, *Phytophthora*, *Pleurotus*, *Polyporus*, *Pycnoporus*, *Rhizopus*, *Sporothrix*, *Talaromyces*, *Trichophyton*, and *Zygorhynchus* produced neither PN-5′-α-G nor PN-4′-α-G in more than 1.5% molar yield.

Screening isolated strains for microorganisms forming PN-α-G

We isolated 53 microorganisms (including 13 fungal strains) from waste water of an industrial plant for PN·HCl and 10 microorganisms (including 4 fungal strains) from contaminated PN-α-G solutions. The proportions of PN-5′-α-G and PN-4′-α-G in cultures of these isolated microorganisms varied widely. One unidentified fungal strain with arthroconidium, named I-1, isolated from the contaminated solution produced PN-5′-α-G with a high molar yield (21%) and high 5′-position selectivity (89%). One bacterial strain, named F-2-1, isolated from waste water, was selected as a microorganism that produced PN-4′-α-G with a high molar yield (24%) and high 4′-position selectivity (92%). The characteristics of strain F-2-1 were as follows; Gram-positive rods (0.8–1.0 × 2.0–3.0 μm), aerobic, spore-forming, non-motile, catalase-positive, oxidase-negative, and O-F-test negative. Strain F-2-1 belonged to *Bacillus*, according to information in Bergey's Manual of Systematic Bacteriology.[10]

Formation of PN-5′-α-G by selected strains

During this survey, strains that yielded PN-5′-α-G with molar conversion of more than 15% (0.75 mM) and that had 5′-position selectivity of more than 75% were selected (Table 2). Of the bacteria, only *Paenibacillus alvei* IFO 3343 had both qualities. Fungi from stock cultures with qualities were limited to 20 strains of only eight species in four genera: *Coriolus*, *Flammulina*, *Schizophyllum*, and *Verticillium*. Four strains in the genera *Coriolus* and *Verticillium* had of 95% and more 5′-position selectivity.

Results of investigation of transglucosylation activity from sucrose and dextrin to 100 mM PN in the culture of eight selected strains in media I and II are shown in Table 3. Medium II gave 1.2- to 10-fold activity per volume of culture medium than medium I. Both activity and 5′-selectivity with *Coriolus fibula* IFO 4949, *Coriolus pubescens* IFO 9782, and *Verticillium dahliae* TPU 4900 were higher than with other strains, even in the presence of this high concentration of PN (100 mM).

Synthesis of PN-5′-α-G by intact cells

Next we examined reactions with these three selected strains harvested from flask cultures with medium II. We used 100 mM PN as the aglycon and dextrin added intermittently as the glucosyl group donor, because preliminary experiments showed cells of these strains could not use sucrose as a glucosyl group donor (results not shown). In the three reaction mixtures, seven unidentified peaks (Fig. 2, peaks A–G) during HPLC of a sample taken after the yield of PN-5′-α-G reached a maximum. Treatment of the reaction mixture with glucoamylase caused a decrease in the area of peak F and an increase in the area of PN-5′-α-G, and all of these peaks were converted to PN by hydrolysis with α-glucosidase (not shown). Terefore, although the peaks have not been identified yet, we presumed that peak F was pyridoxine 5′-α-maltoside, and that the other peaks were other PN glycosides, such as pyridoxine 3-α-D-glucoside and pyridoxine 4′,5′-α-diglucoside.[7]

With *C. fibula* IFO 4949 and *C. pubescens* IFO

Table 2. Microorganism with High 5′-Position-selective Glucosylation

	Culture time (d)	Culture temp. (°C)	Compound found in culture (mM)		5′-Position selectivity (%)
			PN-4′-α-G	PN-5′-α-G	
Bacterium					
Paenibacillus alvei IFO 3343	6	30	0.27	1.56	85.4
Fungi					
Coriolus consors IFO 8348	6	25	0.10	0.85	89.9
C. consors IFO 8726	6	25	0.06	0.82	93.6
C. fibula IFO 4949	6	25	0.04	0.88	95.8
C. hirsutus IFO 6477	6	25	0.04	0.96	95.5
C. hirsutus IFO 6478	6	25	0.17	1.82	91.3
C. pubescens IFO 9782	5	25	0.20	2.52	92.5
C. versicolor IAM 13013	4	25	0.03	0.97	96.7
C. versicolor IFO 4937	5	25	0.08	0.84	91.2
C. versicolor IFO 4941	5	25	0.07	1.02	93.4
C. versicolor IFO 4942	5	25	0.09	0.87	90.7
C. versicolor IFO 6481	6	25	0.16	1.61	91.0
C. versicolor IFO 6482	6	25	0.09	0.85	90.9
C. versicolor IFO 7047	7	25	0.14	2.47	94.6
C. versicolor IFO 8754	6	25	0.12	1.40	92.0
Flammulina velutipes TPU 4674	4	25	0.51	1.89	78.8
Schizophyllum commune TPU 4434	3	30	0.15	1.07	87.7
S. commune IAM 9006	4	25	0.09	0.83	90.2
Verticillium dahliae TPU 4900	4	30	0.07	1.79	96.0
V. dahliae JCM 9509	4	25	0.09	1.16	92.8
V. dahliae JCM 9510	3	25	0.10	1.12	92.1
Isolated fungus I-1	4	25	0.13	1.05	88.8

Table 3. PN-5′-α-G-forming Activity of Selected Strains

Strains were grown aerobically on 5 mL of medium I or II in a test tube. The reaction was done at 40°C for 8 h in the dark with shaking in a assay mixture consisting of 100 mM potassium phosphate buffer (pH 6.5), containing 24.7 mg (0.12 mmol) of PN·HCl, 24 mg of dextrin, 24 mg of sucrose, and 1.0 mL of culture broth, in a total volume of 1.2 mL. One unit of activity was defined as the activity needed to produce one μmol of PN-5′-α-G per minute under these assay conditions.

	Strain	Medium	Culture temp. (°C)	Culture time (d)	Yield of wet cells (mg/mL)	Activity (U/L)	5′-Position selectivity (%)
Bacterium	*Paenibacillus alvei* IFO 3343	I	30	6	14.9	12.2	83.8
		II	30	6	34.1	19.6	84.4
Fungi	*Coriolus fibula* IFO 4949	I	25	4	5.5	3.4	95.8
		II	25	4	29.8	37.0	95.5
	C. pubescens IFO 9782	I	25	11	16.7	7.7	93.4
		II	25	4	33.0	41.6	93.6
	C. versicolor IAM 13013	I	25	4	3.1	3.3	93.7
		II	25	4	12.9	8.0	93.9
	Schizophyllum commune TPU 4434	I	25	6	6.7	3.8	80.3
		II	25	6	15.5	5.7	87.6
	S. commune IAM 9006	I	25	6	27.3	3.0	88.9
		II	25	6	80.6	8.0	88.3
	Verticillium dahliae TPU 4900	I	25	4	33.5	1.5	90.0
		II	25	4	42.7	25.4	89.1
Isolated fungus	I-1	I	25	4	6.9	11.2	92.3
		II	25	4	24.5	13.4	91.7

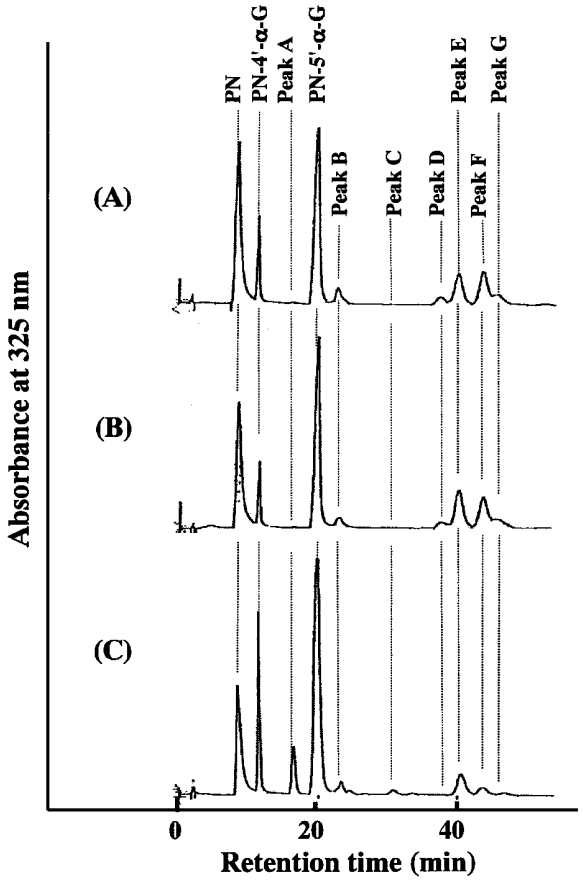

Fig. 2. HPLC of Reaction Mixtures with Intact Cells of Selected Strains.

HPLC was done as described in Materials and Methods. Reaction mixtures after 96 h of incubation with intact cells of *C. fibula* IFO 4949 (A), *C. pubescens* IFO 9782 (B), and *V. dahliae* TPU 4900 (C) were analyzed.

9782, the yield of PN-5′-α-G increased during the reaction between zero to 20 h; the yield was 27% and 30%, respectively, by 20 h, and remained there later, even when more dextrin and cells were added (Fig. 3). *V. dahliae* TPU 4900 produced PN-5′-α-G from PN within 70 h in the highest molar yield, 42%, of these three strains. The 5′-position selectivities of *C. fibula* IFO 4949, *C. pubescens* IFO 9782, and *V. dahliae* TPU 4900 were more than 90% in the early stage of the reaction, but they decreased gradually to 81%, 86%, and 80%, respectively, by 70 h.

The amounts of by-products (not including PN-4′-α-G) were different with each strain tested. In the reaction mixtures with *C. fibula* IFO 4949 and *C. pubescens* IFO 9782 at 70 h, the total amount of by-products was 59% and 73%, respectively, of the amount of PN-5′-α-G, and the total amount of by-products was 30% of PN-5′-α-G with *V. dahliae* TPU 4900. Thus, *V. dahliae* TPU 4900 gave the highest yield of PN-5′-α-G with the smallest amount of by-products.

Formation of PN-4′-α-G by selected strain

Strains that yielded PN-4′-α-G in more than 15% molar conversion (0.75 mM) with more than 75% 4′-position selectivity are listed in Table 4. They were distributed more widely than 5′-position selective strains: there were 11 strains in four genera of bacteria, one actinomycetes strain, one yeast strain, and six strains in three genera of fungi. Some microorganisms belonging to *Verticillium* had 4′-position selectivity, although other strains of the same genus had high 5′-position selectivity.

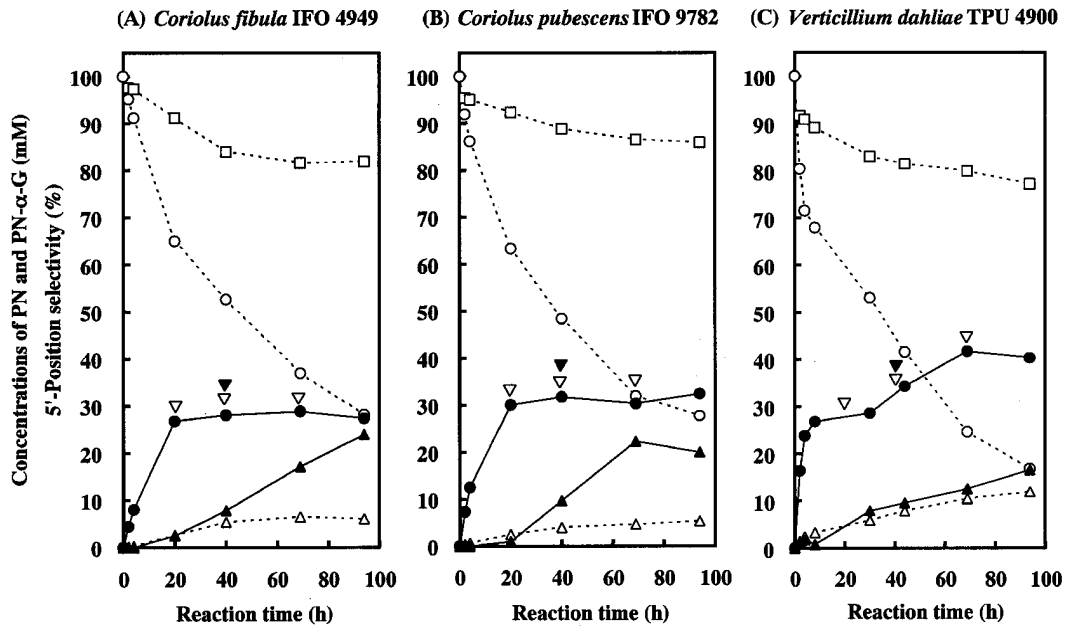

Fig. 3. Changes with Time in PN-5′-α-G Synthesis by Intact Cells of Selected Strains.

The reactions were done as described in Materials and Methods. Symbols: ○, PN; ●, PN-5′-α-G; △, PN-4′-α-G; ▲, total amount of by-products (not including PN-4′-α-G); □, 5′-position selectivity; ▽, addition of dextrin; ▼, addition of cells.

Table 4. Microorganisms with High 4′-Position-selective Glucosylation

	Culture time (d)	Culture temp. (°C)	Compound found in culture (mM)		4′-Position selectivity (%)
			PN-4′-α-G	PN-5′-α-G	
Bacteria					
Bacillus cereus TPU 5504	4	30	0.92	0.04	96.0
B. cereus TPU 5543	3	30	0.86	0.11	88.6
B. megaterium TPU 5522	4	30	1.34	0.17	88.9
B. megaterium TPU 5525	3	30	0.98	0.29	77.4
Edwardsiella hoshinae TPU 6101	4	30	0.84	0.04	95.9
Ochrobactrum anthropi TPU 6850	4	30	0.89	0.06	93.9
Serratia marcescens TPU 7300	4	30	1.03	0.07	93.5
S. marcescens TPU 7302	4	30	1.24	0.33	79.0
S. marcescens TPU 7303	4	30	1.12	0.18	85.9
S. marcescens TPU 7309	4	30	1.26	0.14	90.4
Isolated *Bacillus* sp. strain F-2-1	3	30	1.18	0.10	92.0
Actinomycetes					
Nocardia flavorosea TPU 3019	5	30	1.01	0.28	78.5
Yeast					
Saccharomyces heterogenicus TPU 1043	7	30	1.37	0.05	96.6
Fungi					
Mortierella sp. TPU 4802	4	30	1.68	0.39	81.3
M. isabellina TPU 4808	4	30	1.00	0.27	78.7
Tritirachium dependens IFO 9390	5	25	1.15	0.30	79.3
T. oryzae IFO 7544	4	25	1.03	0.27	79.4
Verticillium fungilcola var. *aleophilum* IFO 30621	5	25	0.83	0.11	88.5
V. niveostratosum IFO 5435	4	25	1.13	0.30	79.0

B. cereus TPU 5504 was one of the strains with highest 4′-position selectivity (96%), so intact cells of this strains cultured with medium I in a flask were examined for PN-4′-α-G synthesis in the same way as for PN-5′-α-G production (Fig. 4). Even at the high PN concentration (100 mM), the strains produced PN-4′-α-G in high yield (33%) and with high 4′-position selectivity (90%) within 19 h. In spite of addition of dextrin and cells at 26 h, the yield of PN-4′-α-G remained almost constant at 30% and the 4′-position selectivity decreased gradually to 80% by 48 h.

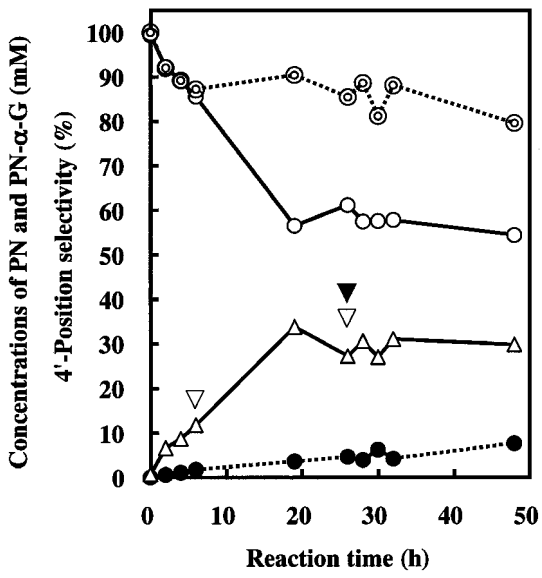

Fig. 4. Changes with in PN-4′-α-G Synthesis by Intact Cells of *B. cereus* TPU 5504.

The reaction was done as described in Materials and Methods. Symbols: ○, PN; ●, PN-5′-α-G; △, PN-4′-α-G; ◎, 4′-position selectivity; ▽, addition of dextrin; ▼, addition of cells.

Discussion

Pyridoxine, often used as one of vitamins of the B_6 complex, is unstable in the light, especially in a neutral solution.[11–13] However, it is almost certain that PN-5′-α-G has both nutritional importance as vitamin B_6 and stability against light and heat, probably because of the masking effect by the glucosyl group at its 5′-position. PN-α-G is be more stable than pyridoxine to irradiation with ultraviolet light and to being heated.[2] The bioavailability of PN-α-G is similar to that of PN, according to results of a long-term feeding experiment with rats.[14] The two positional isomers of PN-α-G enter liver cells to a similar extent. However, during a 60-min incubation with rat liver cells, 78% of PN-5′-α-G is hydrolyzed, but only 15% of PN-4′-α-G is metabolized.[4]

It is necessary for the industrial production of PN-5′-α-G to introduce a glucosyl group into PN anomer selectively and the 5′-position selectively, because of difficulties in separating PN-5′-α-G from PN-4′-α-G,

with their similar physical characteristics. Therefore, an enzymatic method would be suitable for such production of PN-5′-α-G, if an enzyme or microorganism that will glucosylate PN with high 5′-position selectivity and high yield can be found.

The main drawback of the first strain found to produce a large proportion of PN-5′-α-G to PN-4′-α-G,[3] *Micrococcus luteus* IFO 3232, was the little PN-α-G produced: yield was about 6.5% from 98 mM PN and 146 mM sucrose in the intact-cells reaction.[5] Another microorganism, *Micrococcus* sp. strain no. 431 was found by screening with a medium including sucrose as the carbon source from 28 genera of culture collections and isolates from nature,[15] the PN-α-G-forming activity of that strain was similar to that of *M. luteus* IFO 3232.

Carbon sources of screening media for transglucosylation activity should be chosen with their several roles in mind; the substance will be not only carbon source for the growth of microorganisms, but also a glucosyl group donor in transglucosylation, and an inducer of transglucosylation activity. We selected sucrose and dextrin as carbon sources of our screening medium for the reasons given below. Some of enzymes catalyzing the formation of PN-α-G can not use sucrose as a glucosyl group donor,[6,7] although many microorganisms can use sucrose as a carbon source. One half of the glucosyl group of maltose seems not to be used by transglucosylation, but it is used as a carbon source in the screening medium for the formation of 4′-*O*-(α-D-glucopyranosyl)-D-pantothenic acid[16] and *l*-menthyl α-D-glucopyranoside.[17] If dextrin or soluble starch were used as the carbon source, some microorganisms might not grow on the screening medium. We obtained novel 5′- and 4′-positon-selective strains that formed PN-α-G not only by using a newly devised screening medium, but also by extensive microbial screening from stock cultures (907 strains from 129 genera) and from nature (63 strains).

The ability of microbial cultures to produce PN-5′-α-G with high 5′-position selectivity was found in several genera of fungi, for example *Coriolus* (*Basidiomycotina*) and *Verticillium* (*Deuteromycetes*). Members of *Coriolus* are called wood-rotting fungi and some species of *Verticillium* are pathogenic to plants. These two genera can degrade structural compounds of plants.[18–21] Therefore, the β-glucosidase activity of these genera has been examined more than the α-glucosidase activity. It is not known whether one enzyme glucosylates PN with 80–90% regioselectivity, or whether other enzymes with different regioselectivity also exist. We are interested also in the function of regioselective transglucosylation of these fungi in nature. Intact cells of *C. fibula* IFO 4949, *C. pubescens* IFO 9782, and *V. dahliae* TPU 4900, catalyzed the synthesis of PN-5′-α-G from 100 mM PN in yields five to eight times (27%, 30%, and 42%, respectively) higher than the previously reported microorganisms *M. luteus* IFO 3232 and with the same or higher 5′-position selectivity (91%, 92%, and 80%, respectively).

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