Participation of Aspartic Acid and Pyrroloquinoline Quinone in Vitamin B₁₂ Production in 
*Klebsiella pneumoniae* IFO 13541

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*Summary* Vitamin B₁₂ production by Gram-negative facultative anaerobic intestinal bacteria, members of the family *Enterobacteriaceae*, was examined. *Klebsiella pneumoniae* IFO 13541 was the most effective strain with regard to such production. The growth of the strain and its production of vitamin B₁₂ depended exclusively on the concentration of yeast extract added to the medium. The yeast extract components required for the stimulation of bacterial growth and or vitamin B₁₂ production were identified as aspartic acid and pyrroloquinoline quinone (PQQ) and the relationship between vitamin B₁₂ production and these two components was examined. The metabolism of aspartic acid in this process was also investigated; the major metabolites were alanine, glutamic acid, and valine. The formation of alanine depended on dehydrogenase, the activity of which was greatly increased with increasing PQQ concentration.

*Key Words* yeast extract components, vitamin B₁₂, pyrroloquinoline quinone, alanine dehydrogenase, aspartic acid

Since the discovery of Rickes et al. that Actinomycetes were able to produce extracellular vitamin B₁₂ (1), the production of vitamin B₁₂ has been studied extensively in Actinomycetes, e.g., in *Streptomyces olivaceus* (2, 3), and in bacteria, e.g., in *Bacillus megatherium* (4), *Propionibacterium shermanii* (5, 6), and *Pseudomonas denitrificans* (7), and in other organisms, e.g., red algae (8) and *Chlorella pyrenoidosa* (9). However, little is known about the production of vitamin B₁₂ by intestinal bacteria.

We investigated the production of vitamin B₁₂ from various carbon sources by Gram-negative facultative anaerobic intestinal bacateria, members of the family *Enterobacteriaceae*. We have previously reported vitamin B₁₂ production by a most efficient strain, in terms of amounts of vitamin B₁₂ produced, namely *Klebsiella pneumoniae* IFO 13541 (10). The growth of the organism, as well as the amounts
of vitamin $B_{12}$ produced, depended exclusively on the concentration of yeast extract added to the medium, and the yeast extract components required were identified as aspartic acid and pyrroloquinoline quinone (PQQ).

In this study, we examined the effects of aspartic acid and PQQ on the production of vitamin $B_{12}$ by Klebsiella pneumoniae IFO 13541; we also investigated the metabolism of aspartic acid in this vitamin $B_{12}$ production.

EXPERIMENTAL

Microorganisms. Seventy-three strains of Enterobacteriaceae belonging to 8 genera were obtained from the Institute for Fermentation Osaka (IFO) and used in this study.

Medium and cultivation conditions. The basal medium consisted of 3% carbon source, 1% (NH$_4$)$_2$SO$_4$, 0.2% K$_2$HPO$_4$, 0.1% KH$_2$PO$_4$, 0.03% MgSO$_4$$\cdot$7H$_2$O, and 0.5% precipitate CaCO$_3$ (pH 7.0). When Polypepton$^\text{II}$ (Nihon Seiyaku), peptone (Mikuni), or Casamino Acids were used as the sole source of carbon, the concentrations of the carbon source and (NH$_4$)$_2$SO$_4$ were 0.5 and 0.3%, respectively. A 4-ml sample of each organism was grown in a test tube at 28°C for several days under anaerobic conditions (1 mg of solid sodium dithionite was added and air was displaced by argon).

Growth measurement. Bacterial growth was estimated by measuring the turbidity with a Hiranuma photometer at a wavelength of 470 or 610 nm.

Determination of vitamin $B_{12}$. Vitamin $B_{12}$ levels in the culture fluid were quantitatively determined by an assay with Escherichia coli Davis 113-3 (11, 12).

Preparation of cell-free extract. The culture medium for Klebsiella spp. consisted of 30 g of sucrose, 10 g of (NH$_4$)$_2$SO$_4$, 2 g of K$_2$HPO$_4$, 1 g of KH$_2$PO$_4$, 300 mg of MgSO$_4$$\cdot$7H$_2$O, 60 $\mu$g of CoCl$_2$$\cdot$6H$_2$O, and 500 mg of yeast extract in 1 liter of tap water (pH 7.0). Klebsiella spp. cultures grown on slants were inoculated into 800 ml of the medium in 1-liter Erlenmeyer flasks and culture was carried out at 28°C for 2 days under anaerobic conditions. Cells were collected by centrifugation at 10,000 $\times$ g for 20 min and washed twice with ice-cold saline solution. The cell pellet, suspended in 1/200 M potassium phosphate buffer (pH 7.2) containing 0.01% 2-mercaptoethanol, was subjected to the action of an ultrasonic oscillator (Kaijo-denki, 19 kHz) for 16 min at below 10°C. The undestroyed cells and debris were removed by centrifugation at 10,000 $\times$ g for 20 min. The supernatant solution thus obtained was used as the cell-free extract.

Enzyme assay. The following enzymes were assayed by routine methods: aspartate kinase, EC 2.7.2.4 (13); aspartate-semialdehyde dehydrogenase, EC 1.2.1.11 (14); homoserine dehydrogenase, EC 1.1.1.3 (15), homoserine kinase, EC 2.7.1.39 (16); l-threonine 3-dehydrogenase, EC 1.1.1.103 (17); l-aminopropanol dehydrogenase, EC 1.1.1.75 (18); alanine dehydrogenase, EC 1.4.1.1 (19); glutamate dehydrogenase, EC 1.4.1.2 (19); valine dehydrogenase, EC 1.4.1.8 (19); oxaloacetate decarboxylase, EC 4.1.1.3 (20); glycerol dehydrogenase, EC 4.2.1.30

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(21); and propanediol dehydratase, EC 4.2.1.28 (21). Transamination between
aspartic and pyruvic or α-ketoglutaric acids, alanine and α-ketoisovaleric acid,
glutamic and pyruvic or α-ketoisovaleric acids were determined by the amounts of
keto acid formed or remaining. Keto acid was determined by the method of
Friedeman and Haugen (22). Protein content was determined by the method of
Lowry et al. (23). Bovine serum albumin was used as the standard protein.

Amino acid analysis. A Hitachi Model 835 amino acid analyzer was used to
identify the components of yeast extract (fractionated on a Sephadex G-25 column)
and the metabolites in the cultures grown in basal medium (carbon source,
d-ribose) containing aspartic acid (125 μg/ml) or not.

Materials. Yeast extract and vitamin B_{12} (cyanocobalamin) were obtained
from Oriental Yeast Industrial Co. and PQQ was obtained from Mitsubishi Gas
Chemical Co. Alanine dehydrogenase from Bacillus subtilis was obtained from
Sigma Chemical Co. All other chemicals used in this study were commercial
products of analytical grade.

RESULTS

Production of vitamin B_{12} by several strains of Enterobacteriaceae

Citrobacter (2 strains), Enterobacter (7), Erwinia (4), Escherichia (24), Klebsiella (11), Proteus (11), Salmonella (4), and Serratia (10) were examined with
regard to cell growth and vitamin B_{12} production on sugars, Polypepton, peptone,
and Casamino Acids as the sole carbon and energy sources in the basal medium.
Several strains belonging to the genera Enterobacter, Escherichia, and Klebsiella
grew on sugars (D-sucrose, D-maltose, D-glucose, D-fructose, D-galactose, and
D-mannose). However, under anaerobic conditions, there was no cell growth of
strains belonging to the genera Proteus, Serratia Citrobacter, Erwinia or Salmonella
on these sugars. Vitamin B_{12} was produced by several strains belonging to the
genera Citrobacter and Klebsiella (Table 1). As shown in Table 1, Klebsiella
pneumoniae IFO 13541 was the most efficient strain with regard to vitamin B_{12}
production.

Utilization of various carbon sources and production of vitamin B_{12} by Klebsiella
pneumoniae IFO 13541

Klebsiella pneumoniae IFO 13541 further investigated to determine its produc-
tion of vitamin B_{12} from various carbon sources in the basal medium. As shown in
Table 2, the organism utilized almost all the types of sugars, sugar alcohols, and
uronic acids tested.

Vitamin B_{12} was produced when sugars but not sugar alcohols and uronic acids
were used as carbon sources.
Table 1: Vitamin $B_{12}$ production by strains of *Enterobacteriaceae* culture.

| Microbe                  | Carbon source | Aerobic conditions | Anaerobic conditions |
|--------------------------|---------------|--------------------|----------------------|
| *Citrobacter freundii*   |               |                    |                      |
| IFO 12681                | Polypepton    | 0.650 0            | 0.300 9.4            |
|                          | Casamino acids| 0.670 0            | 0.290 10             |
| *Citrobacter freundii*   | Casamino acids| 0.700 0            | 0.360 5.2            |
| IFO 13544                |               |                    |                      |
| *Klebsiella pneumoniae*  | Starch        | 1.500 0            | 0.380 4.6            |
| IFO 3318                 |               |                    |                      |
| *Klebsiella pneumoniae*  | Starch        | 0.860 0            | 0.700 74             |
| IFO 13541                |               |                    |                      |
| *Proteus vulgaris*       | Polypepton    | 0.387 4.4          | 0 0                  |
| IFO 3045                 |               |                    |                      |

The composition of the medium and the culture conditions are described in EXPERIMENTAL, except that, under anaerobic conditions, culture was carried out for 3 days. Under aerobic conditions, culture was carried out for 2 days while shaking at 280 rpm.

Table 2: Vitamin $B_{12}$ production from various sugars, sugar alcohols and uronic acids by *Klebsiella pneumoniae* IFO 13541 in culture.

| Substance    | Growth (OD$_{470}$) | $B_{12}$ (µg/liter) | Substance    | Growth (OD$_{470}$) | $B_{12}$ (µg/liter) |
|--------------|----------------------|---------------------|--------------|----------------------|---------------------|
| Stachyose    | 0.84                 | 96                  | Arabinose    | 0.85                 | 22                  |
| Raffinose    | 0.95                 | 28                  | Xylose       | 0.45                 | 75                  |
| Trehalose    | 1.05                 | 23                  | Ribose       | 0.95                 | 67                  |
| Cellobiose   | 1.41                 | 170                 | Rhamnose     | 0.86                 | 41                  |
| Lactose      | 1.15                 | 63                  | Dulcitol     | 0.55                 | 0                   |
| Sucrose      | 1.10                 | 31                  | Mannitol     | 0.92                 | 18                  |
| Maltose      | 1.20                 | 19                  | Sorbitol     | 0.90                 | 8                   |
| Sorbose      | 0.70                 | 0                   | Arabitol     | 0.76                 | 6                   |
| Mannose      | 1.25                 | 16                  | Inositol     | 0.84                 | 10                  |
| Glucose      | 1.19                 | 18                  | Glucuronate  | 0.93                 | 0                   |
| Fructose     | 0.92                 | 24                  | Galacturonate| 0.85                 | 0                   |

The composition of the medium was the same as that described in EXPERIMENTAL, except that carbon sources were varied as shown in the table. Culture was carried out for 3 days.

*Effects of the yeast extract components PQQ and amino acids on vitamin $B_{12}$ production*

In a previous paper (10), we reported the effects of adding various concentrations of one or the other of yeast extract, meat extract, or CSL (these additives not
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Fig. 1. Effects of each of the amino acid yeast extract components on the growth of and production of vitamin B₁₂ by a strain of Klebsiella pneumoniae IFO 13541. "None" refers to the basal medium described in EXPERIMENTAL. The concentration of each amino acid added to the medium was 125 µg/ml.

Metabolites derived from aspartic acid

These findings suggest that aspartic acid plays a role as a precursor in vitamin B₁₂ production. To characterize the metabolism of aspartic acid, the culture was grown on the basal medium with and without the addition of aspartic acid (125 µg/ml). The metabolites (amino acids) in the culture fluid were routinely extracted.
Table 3. Stimulatory effects of aspartic acid and PQQ on vitamin B₁₂ production and growth of *Klebsiella pneumoniae* IFO 13541.

| Addition                               | Growth (OD₆₁₀) | B₁₂ (μg/liter) |
|----------------------------------------|----------------|----------------|
| None                                   | 0.240          | 30             |
| PQQ 250 pg/ml                          | 0.340          | 97             |
| Asp 125 μg/ml                          | 0.540          | 160            |
| Asp 125 μg/ml + PQQ 250 pg/ml          | 0.560          | 258            |
| Yeast ext. 0.02%                       | 0.460          | 205            |
| Yeast ext. 0.05%                       | 0.565          | 260            |
| Yeast ext. 0.1%                        | 0.622          | 310            |

"None" refers to the basal medium described in EXPERIMENTAL, except that ribose was used as the sole source of carbon. Culture was carried out for 3 days.

Fig. 2. Amino acid analysis of the culture fluid of *Klebsiella pneumoniae* IFO 13541 grown on basal medium to which aspartic acid has been added. Culture was carried out for 3 days. The carbon source was ribose and the aspartic acid concentration was 125 μg/ml.

and subjected to analysis with the amino acid analyzer. Figure 2 shows the results of this analysis; the major metabolites were found to be alanine, glutamic acid, and valine. Alanine was accumulated by about 30–40 μg/ml in the early to middle logarithmic phase, followed by a decline after the maximum growth, whereas valine was increased along with the culture days (26 μg/ml, after 3 days culture). How-
Table 4. Enzyme activity related to aspartic acid metabolization.

| Enzyme                         | Activity (nmol/min/mg) |
|--------------------------------|------------------------|
| Aspartate kinase                | 0                      |
| Aspartate-semialdehyde dehydrogenase | 0                      |
| Homoserine dehydrogenase       | 0                      |
| Homoserine kinase              | 0                      |
| Threonine dehydrogenase        | 0                      |
| Aminopropanol dehydrogenase    | 3.5                    |

Preparation of the enzyme solution and the assay for enzyme activity were the same as those described in EXPERIMENTAL.

ever, no formation of these metabolites was observed in the culture fluid grown on the basal medium without addition of aspartic acid.

Enzyme activity involved in aspartic acid metabolism

As indicated above, aspartic acid and PQQ exerted stimulatory effects on bacterial growth and/or on vitamin B₁₂ production. We therefore examined the relationship between these agents and vitamin B₁₂ production. Aspartic acid participates as a precursor for 1-aminopropan-2-ol in the biosynthetic pathway of vitamin B₁₂. Enzyme activity related to the metabolization of aspartic acid to form 1-aminopropan-2-ol via homoserine and threonine was therefore examined in cell-free extract. The results are shown in Table 4. The activity of aminopropanol dehydrogenase, the enzyme catalyzing the formation of 1-aminopropan-2-ol from aminoacetone, was detected in this organism. However, there was no detectable activity of threonine biosynthesis from aspartic acid. This finding supported the finding of lack of formation of homoserine or threonine from aspartic acid in the cultures as shown in Fig. 2. The metabolites derived from aspartic acid, as stated above, were predominantly alanine, glutamic acid, and valine. The activity of dehydrogenases and aminotransferases involved in the formation of these metabolites was then examined. Table 5 shows the enzyme activity in the metabolism of aspartic acid to form valine. The findings can be summarized thus: 1) aspartic acid is dehydrogenated to form oxaloacetic acid as a coenzyme of NADP, but is not decarboxylated to form pyruvic acid; 2) oxaloacetic acid is decarboxylated to form pyruvic acid; 3) pyruvic acid is then metabolized to form alanine by NADP-dependent alanine dehydrogenase and/or by aminotransferase with aspartic acid or glutamic acid; 4) valine is formed by the transamination reaction between 2-ketoisovalerate and alanine, but not by dehydrogenation; 5) glutamic acid is formed by due to the action of NADPH-dependent dehydrogenase. The effect exerted by PQQ on dehydrogenase activity was found only in the formation of alanine; alanine dehydrogenase activity increased greatly with increasing PQQ concentration (Fig. 3).
Table 5. Enzyme activity related to aspartic acid metabolism.

| Reaction system                        | Activity (nmol/min/mg) |
|----------------------------------------|------------------------|
| Aspartate decarboxylase                | 0                      |
| Aspartate + NAD                        | 0                      |
| Aspartate + NAD + PQQ                  | 0                      |
| Aspartate + NADP                       | 2.45                   |
| Aspartate + NADP + PQQ                 | 2.45                   |
| Oxaloacetate decarboxylase             | 4.68                   |
| Oxaloacetate decarboxylase + PQQ       | 4.68                   |
| Pyruvate + NADH                        | 6.15                   |
| Pyruvate + NADH + PQQ                  | 7.35                   |
| Pyruvate + NADPH                       | 0                      |
| Pyruvate + NADPH + PQQ                 | 0                      |
| Pyruvate-aspartate transaminase        | 2.06                   |
| Pyruvate-glutamate transaminase        | 4.38                   |
| 2-Ketoisovalerate + NADH               | 0                      |
| 2-Ketoisovalerate + NADH + PQQ         | 0                      |
| 2-Ketoisovalerate + NADPH              | 0                      |
| 2-Ketoisovalerate + NADPH + PQQ        | 0                      |
| 2-Ketoisovalerate-alanine transaminase | 3.18                   |
| 2-Ketoisovalerate-glutamate transaminase | 0.88                |
| α-Ketoglutarate + NADH                 | 0                      |
| α-Ketoglutarate + NADH + PQQ           | 0                      |
| α-Ketoglutarate + NADPH                | 0.25                   |
| α-Ketoglutarate + NADPH + PQQ          | 0.25                   |
| α-Ketoglutarate-aspartate transaminase | 0                      |

Preparation of the enzyme solution and the assay for enzyme activity were the same as those described in EXPERIMENTAL. PQQ concentration was 250 pmol/ml.

Fig. 3. Effects of PQQ concentration on alanine formation by dehydrogenation.

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DISCUSSION

Figure 4, which is based on the findings above, shows the metabolism of aspartic acid in the vitamin B$_{12}$ biosynthesis of *Klebsiella pneumoniae* IFO 13541. The resulting succinyl-CoA might convert to form 5-aminolevulinic acid in the structure of the pyrrole ring in vitamin B$_{12}$. In addition, succinyl-CoA also can be considered to be formed from α-ketoglutaric acid by TCA cycle enzymes. As shown in Table 5 and Fig. 3, the enzyme activity of alanine and glutamic acid dehydrogenases was 6.15 (NADH) and 0.25 (NADPH), respectively. PQQ had a remarkable effect on alanine dehydrogenase activity, thus being increased 6- to 7-fold with the addition of 1 μM PQQ. This PQQ effect on dehydrogenase reaction suggests a catalytic action but not that of a coenzyme.

The effects of aspartic acid and PQQ on cell growth and/or on vitamin B$_{12}$ production were examined with 10 other strains of *K. pneumoniae* from type cultures of the Institute for Fermentation Osaka (IFO). Similar results were obtained with 3 type cultures of *K. pneumoniae*, IFO 3318, IFO 12059, and IFO 12932. However, B$_{12}$ yields with these cultures were one-fifth to one-tenth of those achieved with *K. pneumoniae* IFO 13541. Further, several precursors of vitamin B$_{12}$ biosynthesis, i.e., 5-aminolevulinic acid, choline, betaine, methionine, glutamine, 1-aminopropan-2-ol, threonine, 5,6-dimethylbenzimidazole, adenine, and riboflavin, were determined for vitamin B$_{12}$ yields on the basal medium (carbon source, sucrose) with addition of PQQ which compared with aspartic acid. Similar results were obtained with methionine, glutamine, threonine, and riboflavin. On the other hand, when the basal medium with added yeast extract (0.05%) was supplemented with 6 ppm of CoCl$_2$, significant production of vitamin B$_{12}$ was observed. Vitamin B$_{12}$ yields with basal medium containing sucrose with or without the addition of CoCl$_2$ were 580 and 110 μg/liter, respectively.

As reported by Albert *et al.* (25), Klebsiella spp. have been isolated from human small intestine, where they have been shown to have the role of vitamin B$_{12}$ production. The intestinal flora of 10 newborns were studied by Benno *et al.* (26), who found Escherichia, Klebsiella, and other genera belonging to the family

![Fig. 4](image-url)
Enterobacteriaceae in the intestine 1 to 2 days after birth. The presence of these bacteria in the intestine is considered to be advantageous. The bacteria disappeared abruptly on the 3rd to 6th day after birth, when the microflora showed a predominance of Bifidobacterium. The percentage of K. pneumoniae in the intestinal flora has been shown to be 0.01% in the flora of babies on mother's milk and 0.3% in the flora of babies on artificial milk (Mitsuhashi et al. (27)). Finegold et al. showed that, in flora of vegetarians, this percentage was 0.05% (28). Little is known about vitamin B₁₂ production by intestinal microorganisms. Teraguchi et al. (29), investigated the production of this vitamin by Bifidobacteria derived from the human intestine. The extracellular B₁₂ yields of 5 species of the bacteria were 0.1 to 0.2 µg/liter. Klebsiella pneumoniae IFO 13541 used in our study extracellularly produced 100- to 1,000-fold more vitamin B₁₂ than the Bifidobacteria. It is noteworthy that K. pneumoniae is not predominant among intestinal microorganisms, but that it is an excellent producer of vitamin B₁₂.

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