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Nutritional Requirements of *Prevotella* sp. Isolated from the Rumen of the Goat

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Abstract The nutritional requirements for *Prevotella* sp. 4PCCNB2 isolated from the rumen of a native goat in Korea and those of the ATCC 19189 strain isolated from the bovine rumen were investigated. The two strains grew well with ammonium sulfate as the sole added nitrogen source. However, neither a complex of amino acids nor casein hydrolysate effectively replaced ammonium sulfate. Biotin, *p*-aminobenzoic acid, and vitamin B_{12} were essential to culture the ATCC 19189 strain. Unlike the ATCC 19189 strain, however, B_{12} was only stimulatory for the growth of the 4PCCNB2 strain. The 4PCCNB2 strain grew well in the basal medium without an individual acid such as acetic acid or valeric acid. In contrast, either acetic or valeric acid was absolutely required for the growth of the ATCC 19189 strain.

Keywords: bovine rumen, growth response, nitrogen sources, volatile fatty acids, vitamin requirements

In the literature, characteristics of some typical rumen bacteria have been extensively reviewed by Stewart and Bryant [1]. *Prevotella ruminicola* (previously classified as *Bacteroides ruminicola*) is one of the most numerous amylolytic rumen bacteria found in ruminants fed on many different diets [2-6]. It ferments a wide variety of carbohydrates and produces volatile fatty acids (VFA) as rumen fermentation products.

Previous authors have studied about the nutritional requirements for *Prevotella* isolated from the bovine rumen. For example, Russell *et al.* [7] reported the effects of substrates concentrations on the maximum growth rates of *Prevotella*, while Pittman and Bryant [8] studied about the nitrogen sources that affected growth. Strains of *Prevotella* were found to utilize ammonia or peptide nitrogen, but very little free amino acid nitrogen [8]. Early studies on the vitamin requirements of rumen bacteria showed that B_{12} appeared to be required for maximal growth [9]. On the other hand, Caldwell *et al.* [2] found that most strains of *Prevotella* required hemin for growth. VFA requirements for other cellulolytic rumen bacteria were also studied by Dehority *et al.* [10].

Various strains of *Prevotella* have been isolated from bovines, ovines, bison, moose, and elk by several investigators [11]. To our knowledge, however, no work has been done on the nutritional requirements for the growth of *Prevotella* strain isolated from goats. In this investigation, nutritional requirements for the growth of the strain isolated from the goat rumen were studied along with those of *Prevotella* strain isolated from the bovine rumen for comparative purposes.

Prevotella sp. 4PCCNB2 used in this study was isolated at our laboratory from the rumen of a native goat in Korea. Characteristics of the strain (VFA production, substrates, and biochemical reactions) were summarized in an earlier publication [12]. *Prevotella ruminicola* ATCC 19189, one of type strains, was obtained from the American Type Culture Collection. The two strains were maintained in the rumen fluid-glucose-cellobiose-starch (RGCS) agar slants as described by Bryant and Robinson [13].

The composition of the basal medium was similar to the one previously reported [9,14]. Table 1 shows the composition used in this study. To determine the specific nutritional requirements for the growth of *Prevotella*, the so-called single-deletion and single-addition experiments were conducted [9,14]. To investigate the nitrogen source requirements, both casein hydrolysate and ammonium sulfate were excluded from the basal medium. Instead, amino acid solutions [15,16] and/or urea were supplemented in the basal medium.

Growth conditions were similar as previous studies [4, 10]. The medium for the rumen bacteria was distributed in 10-mL amounts in CO_2-gassed 25-mL test tubes, closed by rubber stoppers and autoclaved at 121°C for 15 min. The vitamin solution was sterilized by membrane filtration and then was aseptically added to the sterile medium. The anaerobic culture technique [17,18] was used throughout the course of this work. The organisms were grown on the RGCS medium overnight and then centrifuged. The pellet was washed, resuspended and

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Table 1. Composition of the basal medium

Components	%
Mineral 1[a]	20.0 (v/v)
Mineral 2[b]	20.0 (v/v)
Resazurin	0.0001 (w/v)
Glucose	0.5 (w/v)
Casein hydrolysate (Vitamin-free)	0.2 (w/v)
Vitamin solution[c]	1.0 (v/v)
VFA solution[d]	0.67 (v/v)
Hemin solution[e]	1.0 (v/v)
Na_2CO_3 (8% solution)	5.0 (v/v)
L-Cysteine·HCl·H_2O (2.5% solution)	4.0 (v/v)
CO_2 gas phase, pH 6.8	

[a] Mineral 1 solution contained (per L): 4.5 g KH_2PO_4
[b] Mineral 2 solution contained (per L): 4.5 g NaCl, 4.5 g $(NH_4)_2SO_4$, 0.25 g $CaCl_2$, 0.25 g $MgSO_4$, 0.1 g $MnSO_4·H_2O$, 0.1 g $FeSO_4·7H_2O$, 0.1 g $ZnSO_4·7H_2O$, 0.01 g $CoCl_2·6H_2O$
[c] Vitamin solution contained (per L): 200 mg pyridoxine, 10 mg *p*-aminobenzoic acid, 200 mg riboflavin, 5 mg folic acid, 200 mg thiamin·HCl, 5 mg biotin, 200 mg nicotinamide, 0.5 mg B_{12}, 200 mg Ca-D-pantothenate.
[d] Volatile fatty acid solution contained (per 100 mL): 20 mL acetic acid, 1.0 mL isobutyric acid, 2 mL isovaleric acid, 1.2 mL valeric acid, 2 mL 2-methylbutyric acid. The pH of the solution was adjusted to 7.0 by the addition of 10 N NaOH.
[e] Hemin solution contained 10 mg of hemin dissolved in 50 mL of ethanol plus 50 mL of 0.05 M NaOH.

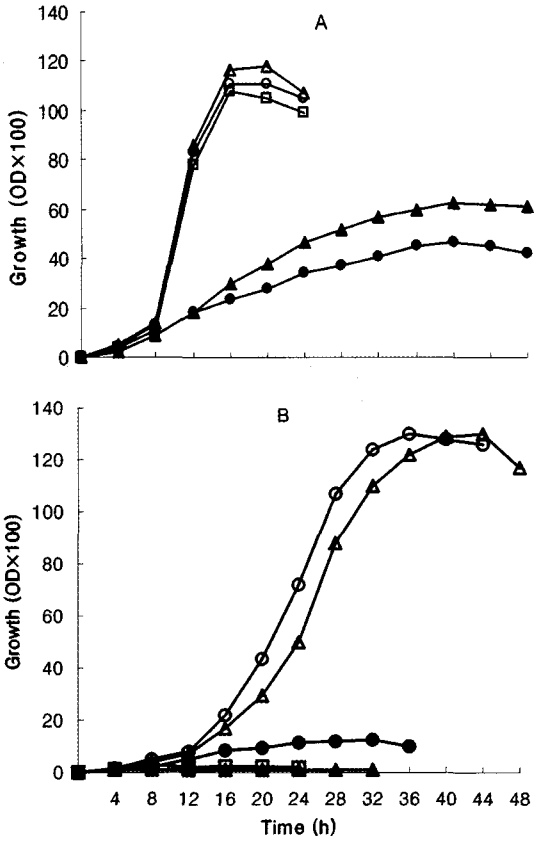

Fig. 1. Growth of the 4PCCNB2 (A) and ATCC 19189 (B) strains in a basal medium, together with the medium minus certain ingredients of the basal medium. Symbols: ○, basal medium; △, minus casein hydrolysate; □, minus volatile fatty acids; ●, minus hemin; ▲, minus vitamins.

then diluted to an optical density (OD) of 0.2 with a specific test medium. Each tube of the test medium was then inoculated with 0.1 mL of the cell suspension. All incubations were carried out at 39°C for up to 2 days under 100% CO_2.

Growth of the cultures was measured by following the increase in optical density at 675 nm in a spectrophotometer (Spectronic 20, Bausch & Lomb). Observation of the colonies on the RGCS agar and Gram stains were used to check for culture contamination from time to time.

The growth of the 4PCCNB2 strain on the basal medium or the medium minus certain ingredients were studied as shown in Fig. 1(A). Both hemin and vitamin B complex were stimulatory in growth to some extent, whereas casein hydrolysate and VFA were not. The presence or absence of casein hydrolysate did not affect the growth, indicating that nitrogen sources, such as ammonium sulfate, were used in the basal medium instead when casein hydrolysate was excluded from the medium. A similar result was reported previously for the growth of *Prevotella* [6,14].

Fig. 1(B) shows the growth of the ATCC 19189 strain under identical conditions. Unlike the 4PCCNB2 strain, the ATCC 19189 strain grew very slowly on the basal medium and demonstrated that an approximately 10 h lag period followed growth. In addition, the ATCC 19189 strain showed mostly absolute requirements for hemin, VFA, and vitamin B complex mixture. It has been previously reported that hemin is essential for the growth of most *Prevotella* strains [2].

Table 2 shows a comparison of the growth responses obtained with the two strains. Compared with casein hydrolysate, ammonium sulfate was an excellent source of nitrogen for the two strains. When ammonium sulfate was present, there was no need for casein hydrolysate. Although the amino acid mixture was slightly stimulatory, neither a complex mixture of 18 amino acids nor urea could replace ammonium sulfate entirely. Either the casein hydrolysate plus the amino acid mixture or ammonium sulfate alone was an adequate nitrogen source. It has been reported that *Prevotella* isolates possess an unusual degree of selectivity of nitrogen sources used for growth [19]. They utilize oligopeptides but cannot utilize much free amino acid nitrogen [8].

As seen in Fig. 1, a mixture of vitamin B complex was required for the growth of both strains. With this in mind, vitamin single-deletion and single-addition experiments

Table 2. Comparison of growth between the 4PCCNB2 and ATCC 19189 strains in a basal medium to which various nitrogen sources were added

Nitrogen source added	Maximal growth (OD × 100)	
	4PCCNB2	ATCC 19189
Casein hydrolysate + $(NH_4)_2SO_4$	120 (16)[a]	128 (36)
Casein hydrolysate	75 (22)	52 (30)
$(NH_4)_2SO_4$	122 (16)	130 (40)
Casein hydrolysate + Amino acid mixture[b] + $(NH_4)_2SO_4$	111 (22)	120 (34)
$(NH_4)_2SO_4$ + Amino acid mixture	119 (22)	125 (36)
Amino acid mixture	53 (34)	32 (18)
Casein hydrolysate + Urea[c]	62 (22)	67 (30)
Urea	21 (22)	23 (18)

[a] Numbers in parentheses indicate numbers of h of incubation required to reach maximal OD.
[b] Amino acid mixture contained (per L): 0.2 g DL-phenylalanine, 0.2 g L-tyrosine, 0.1 g DL-tryptophan, 0.2 g L-aspartic acid, 0.4 g DL-threonine, 0.5 g L-lysine·HCl, 0.6 g L-glutamic acid, 0.5 g L-arginine·HCl, 0.12 g L-histidine·HCl, 0.2 g DL-methionine, 0.2 g L-proline, 0.1 g L-cysteine·HCl, 0.1 g DL-serine, 0.2 g glycine, 0.4 g DL-alanine, 0.5 g DL-valine, 0.5 g DL-leucine, 0.5 g DL-isoleucine.
[c] Urea contained 1.2 g/L.

Table 3. Vitamin single-deletion and single-addition experiments with the 4PCCNB2 and ATCC 19189 strains

Vitamin	Maximal growth (OD × 100)			
	4PCCNB2		ATCC 19189	
	Single addition	Single deletion	Single addition	Single deletion
Pyridoxine	63 (44)[a]	110 (16)	2 (48)	136 (40)
Riboflavin	75 (44)	108 (16)	2 (48)	135 (40)
Thiamin	68 (44)	113 (16)	2 (48)	136 (40)
Nicotinamide	66 (44)	110 (16)	3 (48)	135 (40)
Pantothenate	66 (44)	108 (16)	1 (48)	135 (36)
P-Aminobenzoic acid	66 (44)	110 (16)	14 (20)	95 (46)
Folic acid	67 (44)	108 (16)	1 (48)	137 (40)
Biotin	66 (28)	110 (16)	6 (20)	81 (36)
B_{12}	109 (16)	77 (28)	5 (20)	100 (36)
All vitamins	110 (16)		136 (36)	
None	66 (44)		2 (20)	

[a] Numbers in parentheses indicate numbers of h of incubation required to reach maximal OD.

were carried out as summarized in Table 3. Except for B_{12}, there was no effect on the growth of the 4PCCNB2 strain with a single addition of a vitamin. The 4PCCNB2 strain required B_{12} for maximal growth. Supplementation of B_{12} alone showed essentially the same growth results as that of a complete mixture of 9 vitamins. This means that B_{12} is the only limiting vitamin for the growth of the 4PCCNB2 strain. It is interesting to note that several *Prevotella* species required B_{12} for propionate production [3]. Results from single-deletion experiments shown in Table 3 also confirmed the role of B_{12}. Since possible replacement of B_{12} by methionine had been previously reported [9], quantitative methionine requirements for growth were investigated with 0.02~0.16 g methionine/L. As shown in Fig. 2, increasing the methionine concentration resulted in an increased rate of growth. However, methionine concentrations greater than 0.08 g/L did not stimulate further growth. At this concentration, the

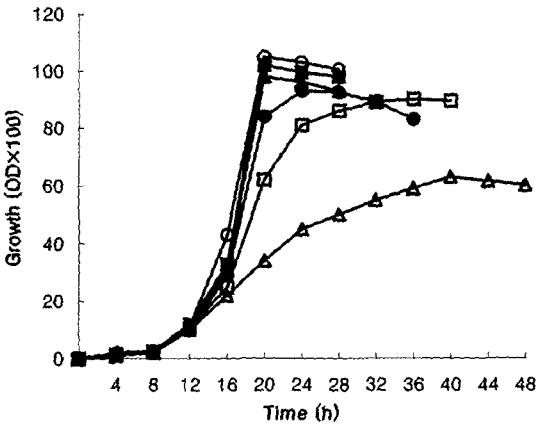

Fig. 2. Effects of methionine concentration on the growth of the 4PCCNB2 strain. Symbols: △, 0.0 g/L; □, 0.02 g/L; ●, 0.04 g/L; ▲, 0.06 g/L; ■, 0.08 g/L; ○, 0.16 g/L.

Table 4. Effect of different vitamins on the growth of the ATCC 19189 strain

Vitamin	Maximal growth (OD × 100)
p-Aminobenzoic acid (PABA)	14 (20)[a]
Biotin	6 (20)
B_{12}	5 (20)
PABA + Biotin	98 (42)
PABA + B_{12}	84 (42)
Biotin + B_{12}	72 (42)
PABA + Biotin + B_{12}	118 (36)
All vitamin mixture	125 (36)
None	2 (20)

[a] Number in parentheses indicate number of h of incubation required to reach maximal OD.

Table 5. Growth responses of the 4PCCNB2 and ATCC 19189 strains to volatile fatty acids

Acid added	Maximal growth (OD × 100)			
	4PCCNB2		ATCC 19189	
	Single-addition	Single-deletion	Single-addition	Single-deletion
Acetic	99 (20)[a]	102 (16)	111 (44)	128 (36)
Isobutyric	110 (16)	109 (16)	3 (16)	132 (32)
Isovaleric	105 (16)	106 (16)	3 (16)	130 (32)
Valeric	105 (16)	106 (16)	130 (40)	128 (32)
2-Methylbutyric	105 (16)	103 (16)	3 (16)	129 (36)
All VFA mixture	108 (16)		132 (36)	
None	101 (16)		2 (16)	

[a] Numbers in parentheses indicate number of h of incubation required to reach maximal OD.

growth rate was almost similar to that of B_{12} supplementation.

Unlike the 4PCCNB2 strain, no appreciable growth for the ATCC 19189 strain occurred in the single-addition experiments. From single-deletion experiments shown in Table 3, however, *p*-aminobenzoic acid (PABA), biotin, and B_{12} were moderately stimulatory. The effects of PABA, biotin, and/or B_{12} on the growth of the ATCC 19189 strain were also studied to demonstrate the extent of vitamin requirements (Table 4). Growth by PABA, biotin and B_{12} was essentially the same as that by a complete vitamin mixture, which contained six additional vitamins. These results suggest that there are some differences in vitamin requirements between the two strains. Thus, vitamin requirements for the 4PCCNB2 strain were found to be minimal.

It is clear from Fig. 1 that the ATCC 19189 strain essentially required VFA mixture for growth but that the 4PCCNB2 strain did not. In Table 5, growth responses of the two strains to various VFA are summarized in detail. As seen in Table 5, the effects of various VFA on the growth of the 4PCCNB2 strain could be neglected. In contrast, one of the straight-chain VFA (*i.e.* acetic or valeric) was found to be essential for the growth of the ATCC 19189 strain. A branched-chain VFA, such as isobutyric, isovaleric, and 2-methylbutyric acid, did not stimulate growth. It is apparent from the single-deletion experiments that the ATCC 19189 strain grew readily with either acetic or valeric acid regardless of branched-chain VFA. Like other cellulolytic rumen bacteria such as *B. succinogenes* [10], VFA requirements differed quite markedly between the two strains.

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