Nutritional Requirements of *Actinomyces* Isolated from Rumen of Goat

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ABSTRACT : The objective of this work was to investigate the nutritional requirements for the growth of *Actinomyces* sp. 9RCC5 isolated from the rumen of a native goat in Korea. The growth of strain 9RCC5 on the basal medium or the medium minus certain ingredients from the basal medium demonstrated that strain 9RCC5 showed absolute requirement of vitamin B complex mixture, while hemin and volatile fatty acids (VFA) were stimulatory to growth to some extent. The 9RCC5 strain grew well with casein hydrolysate as the sole added nitrogen source. However, neither a complex of 18 amino acids nor ammonium sulfate effectively replaced casein hydrolysate. Vitamins such as riboflavin and pantothenate were essential for growth, while thiamin and biotin were stimulatory. With regard to VFA, the growth was stimulated by acetic acid but inhibited by valeric acid. Relatively large quantities of Na^+, K^- and Ca^{2-} were absolutely required for growth. Supplementation of clarified rumen fluid to the basal medium in a range of 0-10% (vol/vol) resulted in an increased rate of growth as well as an increased extent of growth. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 1 : 61-65)***

Key Words : Goat Rumen, Growth Response, Nitrogen Sources, Volatile Fatty Acids, Vitamin Requirements

INTRODUCTION

A variety of rumen bacteria have been isolated using media for counting cellulolytic, amylolytic, proteolytic, lipolytic and methanogenic bacteria, and characteristics of some typical rumen bacteria have been extensively reviewed by Stewart and Bryant (1988). Among cellulolytic rumen bacteria, several genera such as *Butyrivibrio, Fibrobacter* and *Ruminococcus* spp. are already known (Krause et al., 1999; Wang and McAllister, 2002; Lee et al., 2003; Sahu et al., 2004). However, an another cellulolytic rumen bacterium identified as *Actinomyces* was isolated, for the first time, from the rumen of a native goat in Korea (Park et al., 1993).

In the literature, nutritional requirements for the growth of various rumen bacteria have been reported. For example, Russell et al. (1979) reported the effects of combinations on maximal growth rates of several rumen bacteria, while Pittman and Bryant (1964) studied peptides and other nitrogen sources for the growth of *Prevotella ruminicola* (previously classified as *Bacteroides ruminicola*). Early studies for vitamin requirements of several cellulolytic rumen bacteria showed that vitamin B_{12} appeared to be required for maximal growth (Scott and Dehority, 1965). On the other hand, volatile fatty acids (VFA) requirements of cellulolytic rumen bacteria were studied by Dehority et al. (1967). Either isobutyric or 2-methylbutyric acid was required for the growth of *Ruminococcus albus*, but a combination of isobutyric and 2-methylbutyric acids appeared to satisfy the growth requirements of *Ruminococcus flavefaciens*. Inorganic and metal-organic growth requirements of the genus *Bacteroides* were also studied by Caldwell and Arcand (1974).

To our knowledge, no work has been done on the growth response studies with *Actinomyces* sp. isolated from goat rumen. In the present investigation, nutritional requirements for the growth of *Actinomyces* sp. were studied to examine the characteristics of the strain in detail.

MATERIALS AND METHODS

Bacterial strain

Actinomyces sp. 9RCC5 used in this study was isolated at our laboratory from the rumen of a native goat in Korea. Preliminary characteristics of the strain (VFA production, substrates, and biochemical reactions) were described in an earlier publication (Park et al., 1993). The rod-shaped strain was found to be a strict anaerobe and did not produce soluble pigment. The strain was maintained in the rumen fluid-glucose-cellobiose-starch (RGCS) agar slants as described by Bryant and Robinson (1962).

Medium and growth conditions

The composition of the basal medium used was similar to that reported previously (Scott and Dehority, 1965; Varel and Bryant, 1974). The medium contained: glucose 0.5% (wt/vol), casein hydrolysate (vitamin-free) 0.2% (wt/vol), KH_2PO_4 0.09% (wt/vol), resazurin 0.0001% (wt/vol), vitamin solution 1.0% (vol/vol), VFA solution 0.67% (vol/vol), mineral solution 20.0% (vol/vol), hemin solution 1.0% (vol/vol), L-cyctein·HCl·H_2O 1.0% (wt/vol), and Na_2CO_3 4% (wt/vol). The vitamin solution contained (per

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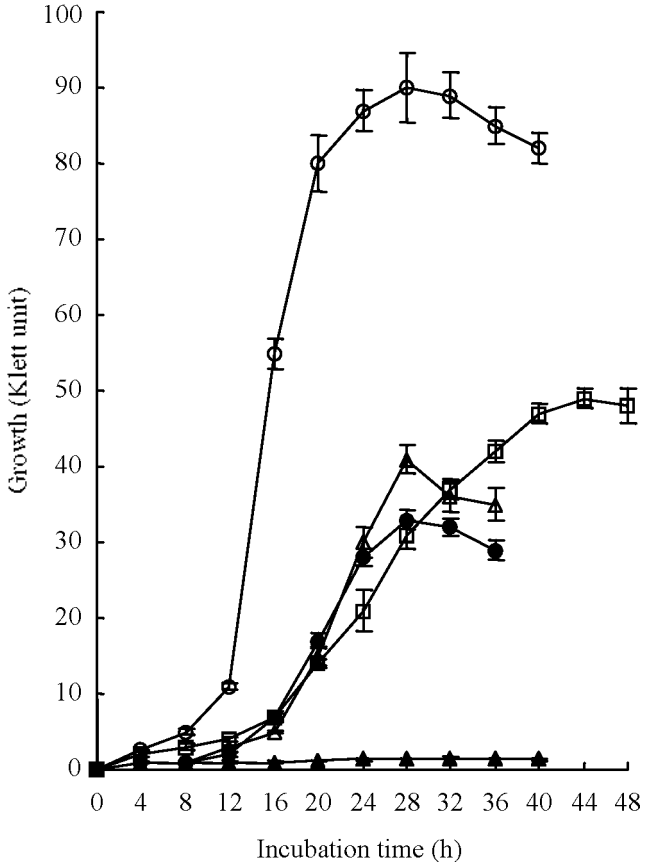

Figure 1. Growth of strain 9RCC5 in a basal medium together with the medium minus certain ingredients. Basal medium (○), minus casein hydrolysate (□), minus VFA (Δ), minus hemin (●), and minus vitamins (▲).

L): pyridoxine, riboflavin, thiamin·HCl, nicotinamide, Ca-D-panthothenate, 200 mg each; *p*-aminobenzoic acid 10 mg, folic acid 5 mg, biotin 5 mg and cobalamin 0.5 mg. The VFA solution contained (per 100 ml): isovaleric acid, n-valeric acid, 2-methylbutric acid, 1.2 ml each; acetic acid 20.0 ml and isobutyric acid 1.0 ml. The pH of the solution was adjusted to 7.0 by the addition of 10 M NaOH. The hemin solution contained 10 mg of hemin dissolved in 50 ml ethanol plus 50 ml of 0.05 M NaOH. The mineral solution contained (per L): NaCl 4.5 g, $(NH_4)_2SO_4$ 4.5 g, $CaCl_2$ 0.25 g, $MgSO_4$ 0.25 g, $MnSO_4 \cdot H_2O$ 0.1 g, $FeSO_4 \cdot 7 H_2O$ 0.1 g, $ZnSO_4 \cdot 7 H_2O$ 0.1 g, and $CoCl_2 \cdot 6 H_2O$ 0.01 g.

To determine the specific nutritional requirements for the growth of strain 9RCC5, the so-called single-deletion and single-addition experiments were conducted (Scott and Dehority, 1965; Varel and Bryant, 1974). To investigate the nitrogen source requirements, casein hydrolysate and/or ammonium sulfate were excluded from the basal medium. Instead, amino acid solutions reported by Linehan et al. (1978) and/or urea (20 mM) were supplemented to the basal medium. Minerals were added to the basal medium in the form of salts for experiments of mineral and nitrogen requirements. Rumen fluid was obtained using the method as reported previously (Leedle and Hespell, 1980). The rumen fluid withdrawn from a rumen-cannulated native goat was strained through two layers of cheesecloth and maintained under O_2-free CO_2 before use. The resulting rumen fluid was used for clarified rumen fluid preparation.

Growth conditions were similar to those reported previously (Dehority et al., 1967; Marounek and Duskova, 1999). The medium for strain 9RCC5 was distributed in 10-ml amount in a CO_2-gassed 50 ml tube having an additional tube (10×100 mm) sealed on obliquely to the side of the tube, closed a rubber stopper and autoclaved at 121°C for 15 min. The vitamin solutions were sterilized by membrane filtration and then were aseptically added to the sterile medium. The anaerobic culture technique (Bryant, 1972) was used throughout the course of this work. The strain was grown on the RGCS medium overnight and then centrifuged. The pellet was washed, resuspended and then diluted to Klett units in a range of 20 to 25 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 statically at 39°C for up to 3 d under 100% CO_2.

Analytical methods

Culture growth was measured during incubation *in situ* by following the increase in Klett unit by using a Klett-Summerson photometric colorimeter (Klett MFG Co., Inc. USA) equipped with a No. 66 red filter (Gordon and Moore, 1961). Culture contamination was checked from time to time by observation of colonies on RGCS agar and Gram stains. All experiments were made in triplicate and replicated at least twice.

Data obtained from these experiments were analyzed using the One-way ANOVA of STATISTIX (STATISTIX, 1996).

RESULTS AND DISCUSSION

Nutritional requirements for growth

The growth of strain 9RCC5 on the basal medium or the medium minus certain ingredients was studied under optimal growth conditions (viz. pH 6.9 and 39°C). As shown in Figure 1, casein hydrolysate appeared to be required for maximal growth since strain 9RCC5 grew very poorly on the medium minus casein hydrolysate. Strain 9RCC5 failed to grow on the medium from which vitamin B complex mixtures were depleted. On the other hand, both hemin and VFA were stimulatory to growth to some extent. It has been previously reported that hemin was required for the growth of some rumen bacteria (Caldwell et al., 1965).

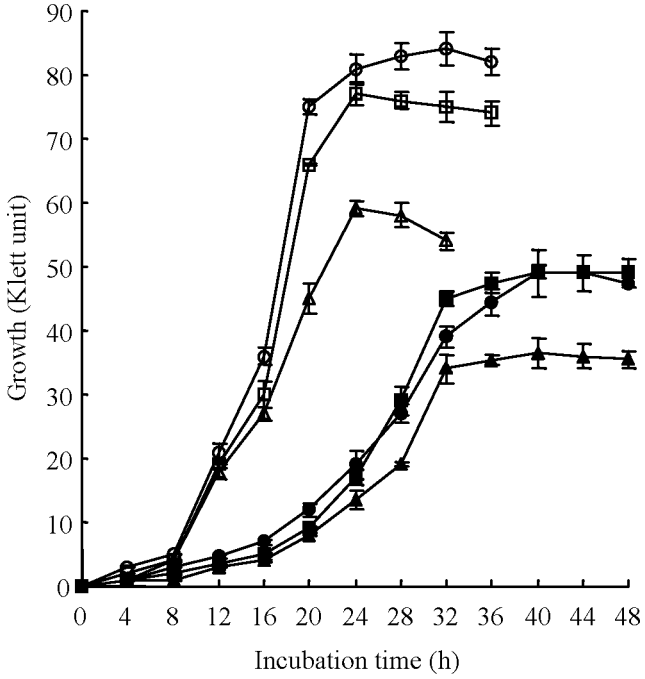

Figure 2. The effect of different nitrogen sources on the growth of strain 9RCC5. The basal medium was the same as that summarized in MATERIALS AND METHOD section, except that casein hydrolysate and ammonium sulphate were excluded. Casein hydrolysate plus ammonium sulphate (○), casein hydrolysate (□), 18 amino acids (△), no addition (●), ammonium sulphate (■) and urea (▲).

Growth responses on various nitrogen sources

Figure 2 shows a comparison of growth responses obtained with various nitrogen sources. The basal medium was the same as that summarized in MATERIALS AND METHODS section, except that casein hydrolysate and ammonium sulfate were omitted. Strain 9RCC5 grew readily with casein hydrolysate as the source of nitrogen and its growth was stimulated further by the addition of ammonium sulfate. Amino acid mixtures were found to be moderately stimulatory, but the presence or absence of either urea or ammonium sulfate did not affect growth. This means that ammonia is not essential as the main nitrogen source. In contrast, amino acids or organic nitrogen sources such as casein hydrolysate are the preferred nitrogen source. It is interesting to note that *Prevotella* isolates possess an unusual degree of selectivity of the nitrogen sources for growth (Madeira and Morrison, 1997). They utilize oligopeptides but cannot utilize significant amounts of free amino acid nitrogen (Pittman and Bryant, 1964).

Vitamin requirements

As can be seen in Figure 1, no appreciable growth for strain 9RCC5 occurred in the medium from which a mixture of vitamin B complex was depleted. With this result in mind, vitamin single-deletion and single-addition experiments were carried out as illustrated in Table 1. Except for riboflavin, there was no effect on the growth of strain 9RCC5 with a single addition of vitamin. Single-deletion experiments summarized in Table 1 confirmed absolute requirements for riboflavin and pantothenate for growth. However, thiamin, *p*-aminobenzoic acid, folic acid, and biotin were moderately stimulatory. Unlike strain 9RCC5, it is interesting to note that biotin is essential for growth of most strains of cellulolytic rumen bacteria such as *R. albus* and *R. flavefaciens* (Bryant and Robinson, 1962). It appears that the degree of biotin requirement for cellulolytic rumen bacteria may be strain-dependent.

Table 1. Single vitamin deletion and single addition experiments with *Actinomyces* sp. 9RCC5

Vitamin	Maximal growth (Klett unit)[a]	
	Single addition	Single deletion
Pyridoxin	2.0±0.2 (68) *	71.2±2.0 (32)
Riboflavin	14.1±0.3 (36) **	3.3±0.2 (68) **
Thiamin	2.5±0.3 (68) *	63.8±1.4 (40) *
Nicotinamide	2.0±0.2 (68) *	74.0±1.3 (32)
Pantothenate	3.0±0.2 (68) **	9.4±0.3 (32) **
p-Aminobenzoic acid	1.5±0.2 (68)	29.5±1.2 (92) **
Folic acid	1.3±0.1 (68)	62.5±1.6 (36) **
Biotin	1.0±0.1 (68)	51.5±1.5 (40) **
Vitamin B_{12}	1.1±0.1 (68)	77.5±3.3 (32)
None	1.0±0.2 (68)	
All vitamin B group		74.3±1.7 (32)

[a] Number in parentheses indicates number of h of incubation required to reach maximal Klett unit.
Values are expressed as the mean±SD (n=3). * $p<0.01$, ** $p<0.001$.

Table 2. Growth responses of *Actinomyces* sp. 9RCC5 to VFA single deletion and addition

Acids added	Maximal growth (Klett unit)[a]	
	Single VFA addition	Single VFA deletion
Acetic acid	72.7±1.9 (24) **	61.8±1.2 (24)**
Isobutyric acid	59.3±1.0 (24) *	82.5±1.4 (24)
Isovaleric acid	52.0±1.3 (30)	87.0±1.6 (20)
Valeric acid	53.5±1.2 (36)	93.0±1.3 (20)*
2-Methylbutric acid	50.8±1.5 (42)	78.5±1.2 (24)*
None	50.0±2.1 (20)	
All VFA		85.3±1.3 (20)

[a] Number in parentheses indicates number of h of incubation required to reach maximal Klett unit.
Values are expressed as the mean±SD (n=3). * $p<0.01$, ** $p<0.001$.

Effect of VFA on growth

It is clear from Figure 1 that VFA are stimulatory to growth to some extent. In Table 2, growth responses of strain 9RCC5 to VFA are summarized in detail. Except for acetic acid, it appears from the single-addition experiments that the effect of VFA on the growth of strain 9RCC5 could be neglected. Results from the single-deletion experiments shown in Table 2 also confirmed the role of acetic acid. This result compares well with the previous result (Dehority et al., 1967). It has been reported that most strains of

Table 3. Effects of ion deletion on the growth yield of *Actinomyces* sp. 9RCC5

Mineral	Growth (Klett unit)[a]
Na^-	5.0±0.7*
K^+	3.5±0.2*
PO_4^{3-}	23.0±1.2*
SO_4^{2-}	56.5±1.5*
Ca^{2+}	5.5±0.3*
Mg^{2+}	17.0±1.8*
Mn^{2+}	58.0±0.8*
Co^{2+}	27.0±1.6*
All minerals	69.0±0.9

[a] Values are expressed as the mean±SD at 48 h incubation (n=3).
* $p<0.001$.

cellulolytic rumen bacteria required acetic acid for growth. In contrast, the absence of valeric acid had influence positively on the extent of growth by about 8% as shown in Table 2. This means that valeric acid inhibits the growth of strain 9RCC5 due to unknown reasons.

Mineral requirements

The effect of mineral solutions on the growth of strain 9RCC5 is summarized in Table 3. It is clear that Na^-, K^- and Ca^{2-} were absolutely required, while $PO4^{3-}$, Mg^{2+} and Co^{2+} were moderately stimulatory. These results compare well with the previous results for the growth of *B. succinogenes* (Bryant et al., 1959). On the other hand, the growth of strain 9RCC5 was not greatly affected by the single deletion of SO_4^{2-}. This may probably due to ability of the strain to utilize the organic sulfur compound such as L-cysteine in the medium (Caldwell and Arcand, 1974). Mn^{2-} deletion from the medium did affect growth to a lesser extent, suggesting that the Mn^{2+} requirement of strain 9RCC5 was partially replaced by other cations or was satisfied by the trace amounts of Mn^{2+} in the medium.

Effect of rumen fluid on growth

A clarified rumen fluid solution was added to the medium in a range of 0 to 40% (vol/vol) and then the effect of rumen fluid on the growth of strain 9RCC5 was studied as shown in Figure 3. The present results showed the growth-promoting activity by the rumen fluid for strain 9RCC5. Similar results were previously reported for most strains of rumen bacteria (Gordon and Moore, 1961; Caldwell et al., 1965). The presence of 10% rumen fluid in the medium resulted in an increased growth rate by 1.2 times as well as an increased extent of growth by 1.6 times. However, the growth was not proportional to the amount of rumen fluid concentrations above 10% supplementation. It is likely that some unidentified components such as growth factors in the rumen fluid provided growth stimulation for strain 9RCC5.

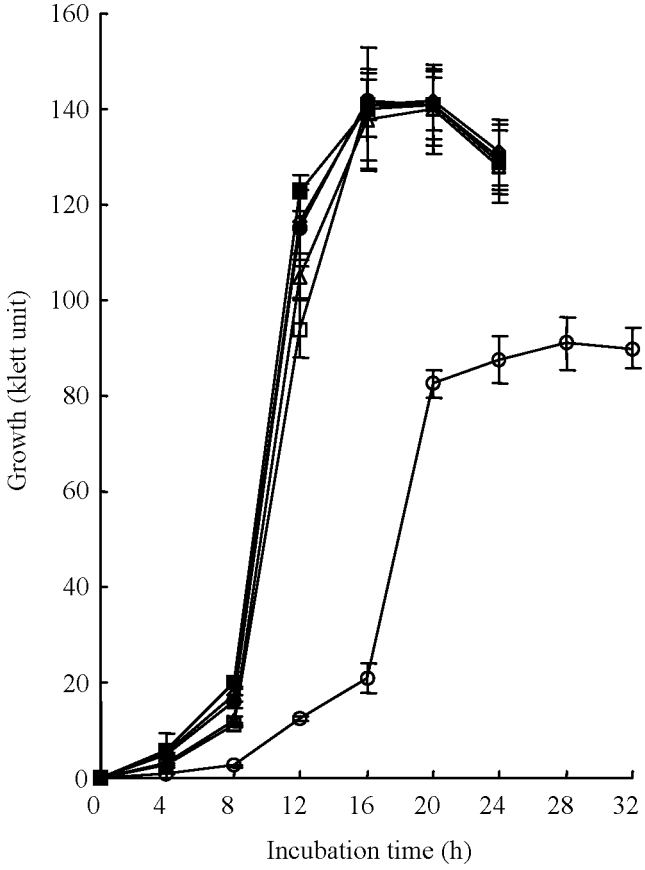

Figure 3. Growth responses of strain 9RCC5 on clarified rumen fluid addition to the basal medium. 0% (○), 10% (□), 20% (Δ), 30% (●) and 40% (■).

CONCLUSIONS

The nutritional requirements for the growth of *Actinomyces* sp. 9RCC5 were investigated in detail. The growth of the 9RCC5 strain showed absolute requirement of vitamin B complex, while hemin and VFA were stimulatory to growth to some extent. The 9RCC5 strain grew well with casein hydrolysate as the sole nitrogen source. Studies on mineral requirements showed that large quantities of minerals such as Na^+, K^+ and Ca^{2+} were absolutely required for growth. It was also evident that supplementation of clarified rumen fluid to the basal medium resulted in an increased rate of growth.

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