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Biotin Requirement for the Growth and Sporulation of *Bacillus subtilis* SNU816 in a Synthetic medium

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Bacillus subtilis SNU816의 合成培地에서의 성장과 포자형성을 위한 Biotin 要求性에 관하여

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ABSTRACT

The effect of biotin on the growth and sporulation of *Bacillus subtilis* SNU816 was investigated. When *B. subtilis* SNU816 was cultured on glucose as a sole carbon source, the growth was retarded markedly and usually ceased at early log phase. But by addition of biotin to this medium, normal, rapid growth was restored. The growth rate was increased proportionally according to the concentration of exogenous biotin until it reached to 0.05 μg/ml, at which about three fold rapid growth was achieved. Also biotin was required for optimum sporulation for it facilitated the complete utilization of both glucose(Glc) and glutamic acid(Glu). Without biotin in Glc+Glu medium, about 40% of glutamic acid was remained unutilized. The dipicolinic acid content of cells cultured in Glc+Glu medium without biotin was markedly small and sporulation was suppressed before free spore release. Since biotin could be partially replaced by one of TCA cycle intermediates such as oxalacetic acid, citric acid, or glutamic acid in enhancing growth in Glc medium, it was postulated that this strain might have a defect in converting pyruvate to oxalacetate which process is known to be mediated by pyruvate carboxylase that requires biotin as a cofactor.

INTRODUCTION

Bacillus subtilis SNU816 is a aerobic, spore-forming bacterium. It is widespread in Korea, and was firstly isolated by Lee(1974). In spite of its importance in fermenting Mae-ju, one of Korean traditional foods, little has been studied to characterize this strain. Since the cultivation of this strain in a synthetic medium containing glucose as a sole carbon source often failed, precise characterization of its physiological properties is required. During experiment, it was revealed that the addition of biotin to the culture medium amended the defect of glucose utilization, and some of carbon sources such as glutamic acid, citric acid, oxalacetic acid could replace biotin in facilitating glucose utilization. Therefore, the effect of biotin on the growth and sporulation of this strain was studied with special interest to the role of biotin in utilization of glucose and glutamic acid for lots of available data concerning to these two compounds had been accumulated (Depamphilis and Hanson, 1969; Hanson and Cox, 1967; Diesterhaft and Freese, 1973; Buono *et al.*, 1966; Bernlohr,

1967; Nickerson *et al.*, 1974; Kennedy *et al.*, 1971, etc).

MATERIALS AND METHODS

Microorganism

B. subtilis SNU816(Lee, 1974) stock-cultured in nutrient agar (Difco) slant was used throughout the experiment. Before using as an inoculum to synthetic test medium, slant cultures were activated by two transfers in fresh nutrient broth maintaining exponontial phase (A_{600} =0.5). Cells from this culture were immediately harvested by centrifugation (1,500×g, 10min) and washed thoroughly with sterile minimal salt culture solution prewarmed to 37°C.

Inadequate washing may induce an incorrect result caused by residual nutrients originated from complex medium. The pellet was resuspended in half original volume of washing solution and was used as inoculum.

Media

Basal minimal salt solution (Elmerich and Aubert, 1975) was used with only minor modification. 2g/*l* of each carbon source was added to minimal salt solution. If carbon sources were added together, then following concentrations were used; glucose(1g)+glutamic acid(1g), glucose(1.8g)+citric acid(0.2g), or glucose (1.8g)+oxalacetic acid(0.2g). Biotin was added to final concentration of 50μg/ml, if needed. The pH of each medium was adjusted to appropriate value and the media were sterilized by membrane filtration(0.45μm Milipore). After sterilization, appropriate volume fractions of each medium were divided into sterile L-shaped test tubes. These tubes were carefully selected to be suitable in measuring turbidity directly with spectrophotometer.

Cultivation

Equal amounts of cells were inoculated into each medium in L-shaped test tube, and incubated at 37°C in air-controlled shaker at a speed of 120rpm.

During cultivation, each test tube was taken out of incubator, and the turbidity was directly measured with spectrophotometer, and the tube was reincubated. By using this method, rapid measurement of turbidity was possible (one sample could be measured within 10sec.), and thus it reduced the loss of time, labour, materials, and possible error caused by delayed sampling and measurement which is critical in determining the growth rate.

Growth was measured by spectronic 20 colorimeter (Bausch & Lomb). Percent sporulation was measured either by counting the refractile cells under phase contrast microscopy (Nikon Apophot Multipurpose microscope with photographying appendage) or by spore stain (Bartholomew and Mittwer, 1950).

pH was measured by pH meter (Fisher Accumet Model 230A pH/Ion Meter).

Glucose was determined by using glucose oxidase system (Boehringer Mannheim GmbH Product).

Glutamic acid was determined by using glutamate dehydrogenase system according to the method described by Erlich and Bergmeyer (1974).

Extracellular protease(ECP) activity was determined by using 0.6% Hammarsten casein as substrate with minor modification of method described by Ogrydziak and Scharf (1982). The enzyme activity was expressed as μmole tyrosine produced per ml of enzyme solution per min.

Dipicolinic acid(DPA) was determined colorimetrically as described by Janssen *et al.* (1958).

Spectrophotometric measurements of absorbances were carried out with Cecil CE272 Linear Readout Ultraviolet Spectrophotometer for DPA and glucose, and with Gilford Spectorophotometer 250 for glutamic acid and ECP, respectively.

Centifugations were carried out with Inter-

national Centrifuge R(size 2) for cell washing, and Hitachi 20PR-5 for cell harvest, respectively.

Chemicals

L-glutamic dehydrogenase sodium salt (50 unit/mg), ADP, β-NAD, hydrazine hydrate, DPA, glycine, D-glucose, and glutamic acid were purchased from Sigma Chemical Co., casein (Hammarsten), from ICN Pharmaceuticals, glucose analysis reagents, from Boehringer Mannheim GmbH, respectively. And all other salts (S.P.C. GR grade) were purchased from Shinyo Pure Chemicals Co.

RESULTS AND DISCUSSION

Effect of Biotin on the growth of *B. subtilis* SNU 816 upon glucose and/or glutamic acid.

Since the growth of *B. subtilis* SNU 816 in a synthetic medium with glucose as a sole carbon source was often failed, the growth in the Glc medium was profiled at the presence or absence of biotin. Since biotin has been reported to be related in glutamic acid metabolism (Hubbard and Hall, 1968), the effect of biotin on the utilization of glutamic acid was investigated together. Another reason was as follows. If biotin would influence the glucose metabolism, it would not engage in glucose catabolism itself but rather indirectly by coupling of glucose catabolism to another anabolic pathway, as it were, coupling of Embden-Meyerhof (EM) pathway to biosynthesis of energy or macromolecules such as protein, lipid, and nucleotides via tricarboxylic acid (TCA) cycle. Therefore one of the substrates that, if catabolized, enter directly into TCA cycle was chosen. Glutamic acid is one of these substrates (Meister, 1965).

However, because these two compounds are different in pH property, experiments were performed at different pH conditions (pH 7.0 and pH 5.0).

The results were shown in Fig. 1. The growths of *B. subtilis* SNU 816 were apparantly facilitated by biotin regardless of kinds of substrate and degree of initial pH. Growth without biotin at Glc medium was trivial at both pH, and caesed at early exponential phase. (Fig. 1a, b; ○——○)

But once biotin was added, the normal, rapid growth was restored (Fig. 1a, b; ●——●). At low pH condition, however, growth was ceased at mid log phase (OD=0.5). This indicates that if glucose is degraded, much of acids may be produced and accumulated in the medium, causing rapid decrease of medium pH (Nakata and Havlorson, 1960).

In general, the growths were delayed as the medium pH was lowered. But only one exception was encountered in Glu+Bio medium. Althcugh the growth rate in Glu medium was decreased as medium pH was lowered, the additicn of biotion to this medium facilitated the grotwh much notably at low pH (Fig. 1b; ■——■). This result implies that glutamic acid utilization system involved or mediated by biotin may be fully activated at low pH or the humble increase in growth at high pH may merely be resulted from suppression of growth by pH up caused by glutamic acid utilization.

Another phenomenon observed here was that by using both glucose and glutamic acid as carbon sources considerable enhancement of growth was achieved even at the absence of biotin (Fig. 1a, b; △——△). This implies that active operations of both EMP and TCA cycle may be required for active growth of this strain and the failure of growth in Glc medium may be partially caused by uncoupling of EMP to TCA cycle. This possibility was investigated later part of this experiment.

At any rate, since biotin seemed to affect considerably the rate of glucose consumption, the effect of biotin concentration on the growth

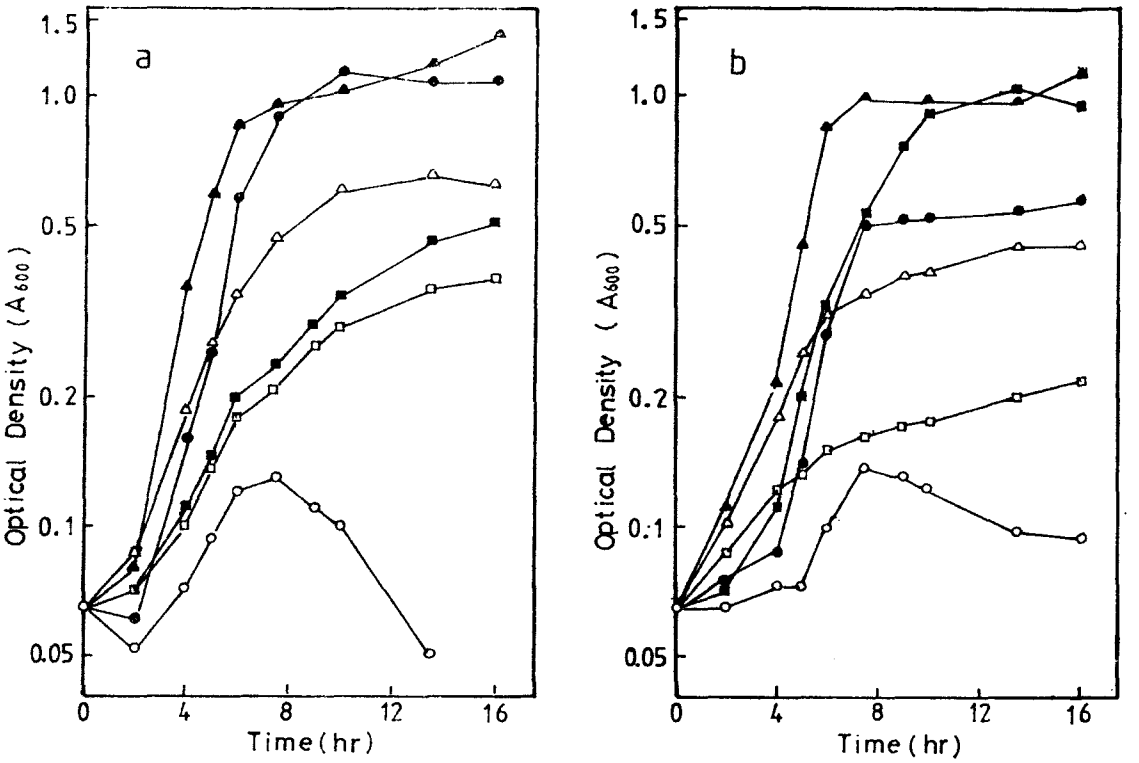

Fig. 1. The effect of biotin on the growth of *Bacillus subtilis* SNU816. Cells were cultured on glucose and/or glutamic acid at different pH condition (a; pH7.0, b; pH5.0) with biotin (closed) or without biotin (open).
○——○ ; glucose, □——□ ; glutamic acid, △——△ ; glucose+glutamic acid

rate in the Glc medium was investigated. As was shown in Fig. 2, growth rate was progressively increased as the concentration of biotin increased. The growth rate was increased up to three fold, culminating to 0.637hr^{-1} as the concentration of biotin reached to 0.05μg/ml. The enhancement of growth by biotin, in fact, may be much more distinct because the growth without biotin was usually ceased at early log phase.

All of these results confirmed that the utilization of glucose as a sole carbon source was quitely dependent on the presence of biotin.

Effect of biotin on the growth and sporulation of *B. subtilis* SNU816 in Glc+Glu medium.

Since the utilization of glucose and glutamic acid were revealed to be related to biotin, direct measurement of substrates remained in the

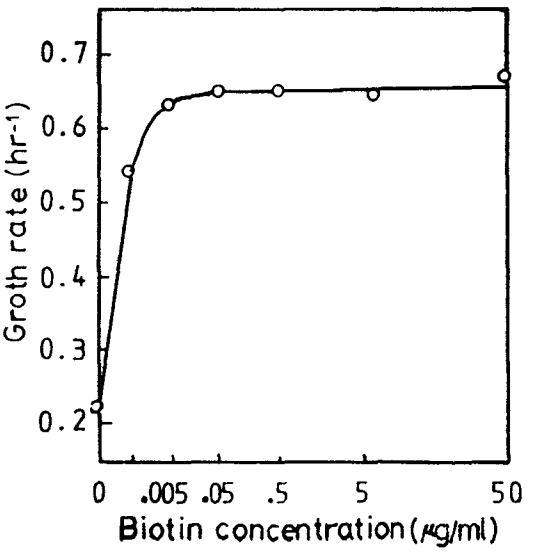

Fig. 2. The effect of biotin on the growth of *B subtilis* SNU816 in Glc medium (pH7.0)

medium was performed during cultivation in Glc + Glu medium with or without biotin. Since the utilization of these substrates seemed to influence the changes of medium pH in the opposite direction, the pH changes of the medium were also tested.

To investigate the influence on sporulation, sporulation specific events such as ECP production, refractivity, and DPA production were investigated. The initial pH of each medium was adjusted to 6.0 because either pH 7.0 or pH 5.0 seemed to be disadvantageous for rapid utilization of glutamic acid or glucose.

As was shown in Fig. 3, growth rates in both Glc+Glu and Glc+Glu+Bio medium were increased as compared with those in media of pH 7.0 or pH 5.0, with same composition. The utilization rates of substrates coincided well with the degree of growth, and the patterns of substrate utilization were similar in both media except that biotin facilitated the consumption of both glutamic acid and glucose. At early growth phase, small portions of glutamic acid were used first and followed by rapid overpassing consumption of glucose. The pH of each medium was decreased rapidly during this phase. It was reported that in most *Bacillus* spp. growing in a glucose-containing medium, the net flow of carbon occurred through the Embden-Meyerhof(EM) pathway in the direction of pyruvate production (Depamphilis and Hanson, 1969), and because the TCA cycle was partially suppressed during exponential growth, the majority of acids produced was remained unutilized and caused rapid pH down of medium (Nakata, 1963). When the glucose was exhausted from the medium, rapid growth was ceased and the utilization rate of glutamic acid was increased accompanying with the increase of medium pH. But the rate of glutamic acid utilization at late growth phase was considerably influenced by the presence of biotin. In biotin-absent medium, glutamic acid was utilized only slowly and considerable amounts were

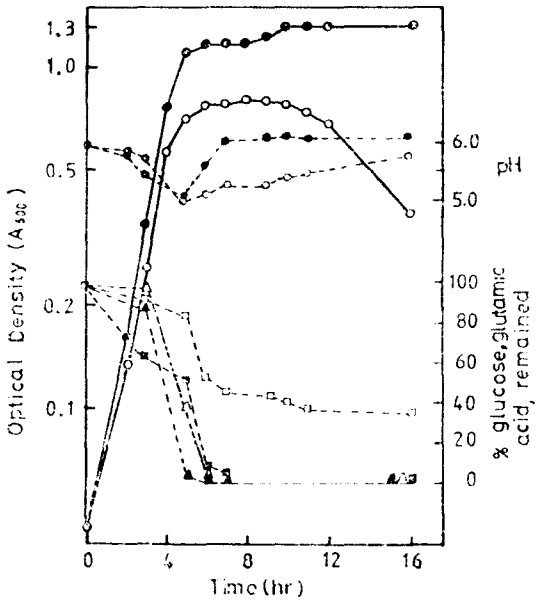

Fig. 3. Effect of biotin on the growth of *B. subtilis* SNU816 in the Glc+Glu medium (pH6.0).
open: without biotin, closed: with biotin
o——o ; bacterial growth, o·····o ; pH
△·····△ ; glucose, □·····□ ; glutamic acid

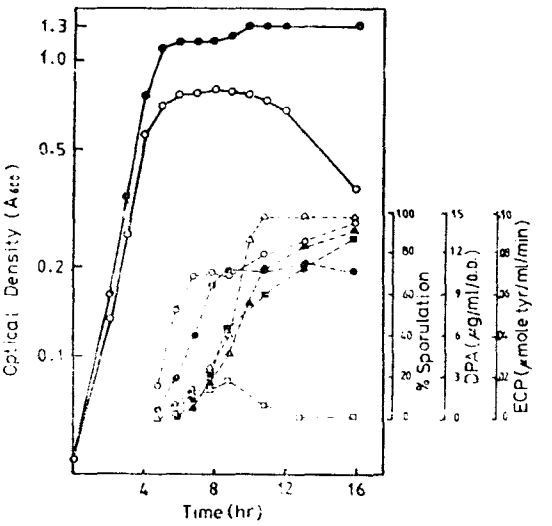

Fig. 4. Effect of biotin on the sporulation of *B. subtilis* SNU816 in the Glc+Glu medium (pH6.0)
open: without biotin, closed: with biotin
o——o ; bacterial growth, o·····o ; ECP activity, △·····△ ; refractivity, □·····□ ; DPA content

remained unutilized throughout (Fig. 3; □·····□). But in biotin-present medium, glutamic acid was utilized rapidly and completely (Fig. 3; ■·····■). These results implied that glucose utilization was not solely dependent on the presence of biotin because the rate of glucose utilization in either Glc+Glu medium or Glc+Glu-Bio medium was almost same, while the utilization of glutamic acid was distinctibly influenced by biotin. It was worth noting that glutamic acid alone could successfully stimulate the utilization of glucose.

Biotin was also required in sporulation process of this strain. Though the refractivity and ECP activity appeared faster in biotin-absent medium (Fig. 4; △·····△, ○·····○, respectively) than in biotin-present medium (Fig. 4; ▲·····▲, ●·····●, respectively), the DPA content in biotin-absent medium (Fig. 4; □·····□) was much less than that in biotin-present medium (Fig. 4, ■·····■). Kennedy *et al.* (1971) reported that glutamic acid was required in the medium before or during forespore and cortex formation to influence DPA content and heat resistance. Therefore, the lack of DPA in the cells cultured in biotin-absent medium seemed to be resulted from the insufficient incorporation of glutamic acid. Furthermore, free spore release was also influenced by biotin. As was shown in Fig. 5, cells cultured in biotin-present medium (Fig. 5a, arrows) produced clear, small free spores, indicating normal sporulation was completed. In contrast, cells cultured in biotin-absent medium (Fig. 5b, arrows) beared some surplus around the spores (hence appeared thick, irregular, and rough), indicating that sporangium had undergone abnormal lysis without completion of sporulation. In reality, cells from this medium were unstable and severely lysed at later stationary phase (Fig. 3; ○——○). The high level of ECP of this abnormal cells might be caused by such lysis, thus the intracellular protease activity was checked together with ECP activity.

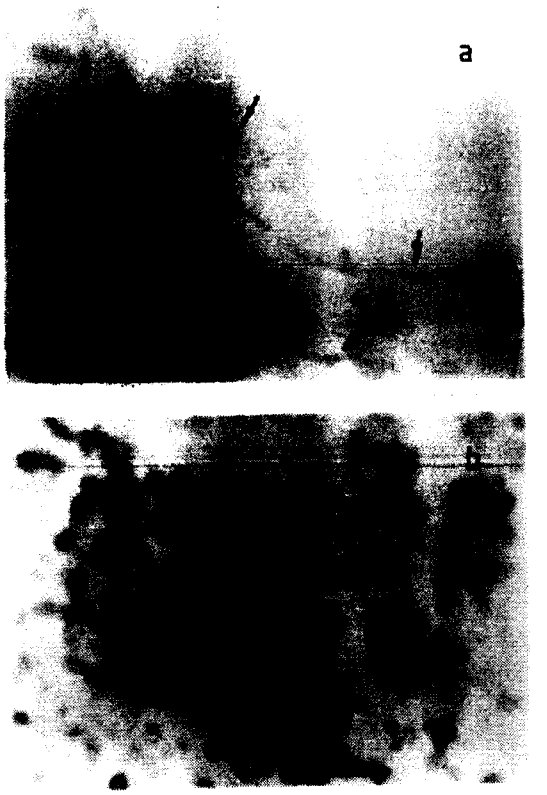

Fig. 5. Photographs of stained spores of *B. subtilis* SNU816 cultured in Glc+Glu medium (pH 6.0) with biotin (a) or without biotin (b). Arrows indicate the free spores. Pictures were taken at 16 hrs of cultivation.

Effect of TCA cycle intermediates on the growth of *B. subtilis* SNU 816

Since the failure of growth in Glc medium was amended by simple addition of glutamic acid or biotin, the possibility that the growth of *B. subtilis* SNU816 might be stimulated by addition of TCA intermediates was investgated. Of the TCA intermediates, oxalacetic acid and citric acid were chosen because they were intermediates of just prior, and posterior site where coupling of EMP to TCA cycle occured.

As table 1 denoted, the substrates which were beyound the coupling site could support good growths even at the absence of biotin, but the substrates before the coupling site couldn't.

Citric acid alone could support good growth

Table 1. Effect of TCA cycle intermediates on the growth of *B. subtilis* SNU816 (at pH 7.0)[a]

Substrate	without biotin		with biotin	
	growth rate	lag period[b]	growth rate	lag period[b]
glc	0.236	11	0.637	7
glu	0.250	7.6	0.295	6.3
cit	0.207	18	0.426	14.8
oxal	no growth	—	no growth	—
glc+glu	0.475	4.5	0.739	3.5
glc+cit	0.544	9.3	0.724	5.1
glc+oxal	0.355	9	0.549	4.5

a: mean value of 3 experimental data.
b: the time(hr) required to reach $A_{600}=0.3$ from initial $A_{600}=0.065$

though long lag period was required. But oxalacetic acid alone couldn't support any growth even at the presence of biotin. The addition of oxalacetic acid to Glc medium, however, enhanced the growth of this strain considerably.

These results suggested that this strain might have a defect in synthesizing oxalacetic acid, resulting in poor operation of TCA cycle.

It was reported that in *B. subtitis* cultured in glucose-containing medium, substrate amounts of oxalacetic acid was required for optimum operation of TCA cycle during exponential phase and that the synthesis of oxalacetate was primarily mediated by a constitutive enzyme, pyruvate carboxylase converting pyruvate to oxalacetate through carboxylation (Diesterhaft and Freese, 1973). Since this enzyme required biotin as a cofactor, it was somewhat clear that the defect of *B.subtilis* SNU816 in synthesizing oxalacetic acid might, in fact, be caused by being defective in synthesizing biotin.

This might be the reason why *B. subtilis* SNU 816 couldn't grow on glucose as a sole carbon source and required biotin for its optimum growth on glucose. But this conclusion was only presumptive and more evidences including measurement of pyruvate carboxylase activity are required.

The experiments to find out such evidences are now being carrid out in this laboratory.

적 요

Bacillus subtilis SNU816의 성장 및 포자형성에 미치는 biotin의 영향에 관해 연구되었다.

B. subtilis SNU816을 glucose를 유일한 탄소원으로 하여 배양하였을 경우 성장은 상당히 지연되었으며, 보통 대수기 초반에서 멈추었다. 그러나 여기에 biotin을 첨가할 경우 정상적인 빠른 성장이 이루어졌다. 이 균의 성장속도는 biotin의 농도가 0.05μg/ml에 이를때까지 비례하여 증가하였으며, 이 경우 약 3배의 빠른 성장이 유발되었다.

또한 biotin은 포자형성시에도 요구되었는데 그것은 biotin이 glucose와 glutamic acid등의 이용을 촉진하였기 때문이다. biotin이 없을 경우 약 40%의 glutamic acid가 이용되지 않은 채 배지에 남아 있었으며 내생포자에 특이한 물질인 dipicolinic acid의 생성이 억제되고 자유 포자의 형성도 억제되었다.

biotin이 글루코오스 배지에서의 이 균의 성장을 촉진시키는 현상은 oxalacetic acid, citric acid, glutamic acid와 같은 TCA 회로의 중간산물에 의해서도 나타나는 것으로 보아 이 균이 비오틴을 요구하는 까닭은 pyruvate를 oxalacetate로 전환시키는 pyruvate carboxylase가 biotin을 요구하기 때문인 것으로 사료되었다.

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