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Effect of Isoleucine on Toxin Production by *Clostridium difficile* in a Defined Medium

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Summary

Supplementation of a carbohydrate-free minimal medium with a high level (100 mM) of histidine, methionine, valine, isoleucine, proline and leucine, in particular isoleucine, markedly increased toxin production by *Clostridium difficile* VPI 10463. The effect of isoleucine was further examined. Increasing the concentration of isoleucine from 20 to 100 mM remarkably increased toxin production, while bacterial growth decreased gradually. Amino acid analysis of the culture revealed that, at 100 mM isoleucine, consumption of isoleucine was remarkably increased. During the incubation period when toxin titers increased markedly but bacterial growth was declining, isoleucine, leucine and cysteine were taken up preferentially and alanine and cystathionine, which were not found at 1 mM isoleucine, were produced in large quantities. These findings suggest that isoleucine may play an important role in toxin production by *C. difficile* and that alanine and cystathionine production may be co-regulated with the toxin production in the absence of fermentable carbohydrates.

Introduction

C. difficile causes pseudomembranous colitis and is a major etiological agent of antibiotic-associated diarrhea (2, 4, 14, 16). Pathogenicity is mainly due to the production of two toxins designated toxin A (enterotoxin) and toxin B (cytotoxin) (1, 29). The properties of these toxins have been extensively studied physicochemically, immunologically and biologically. Recently, it was demonstrated that both toxins, A and B, are monoglucosyltransferases that

selectively modify the low-molecular-mass GTP-binding proteins of the Rho subfamily (11, 12). The cosubstrate UDP-glucose is cleaved by the toxins, and the glucose moiety is transferred to amino acid threonine 37 of Rho, which is located in the effector domain of the Rho proteins (11, 12, 28).

Analysis of the regulation of toxin production is also important to explain pathogenicity of *C. difficile*. However, even the nutritional requirements for toxin production have not yet been fully understood. It has been reported that not all of the amino acids required for bacterial growth are essential for toxin production (8, 30). As to vitamin requirements, it has been demonstrated that biotin insufficiency enhances toxin production (31). While *C. difficile* is of both saccharolytic and asaccharolytic nature, most studies have been performed in the presence of fermentable carbohydrates and little is known about the specific role of various essential nutrients for toxin production in their absence.

In the present study, we investigated the effects of amino acids, particularly isoleucine, on toxin production in the absence of fermentable carbohydrates, using a recently developed defined medium (13).

Materials and Methods

Strains

Toxigenic *C. difficile* VPI 10463 was used throughout this study.

Media

A defined medium, *C. difficile* minimal medium (13) without glucose [G(–)CDMM] was used as a basal medium. The concentrations of amino acids in G(–)CDMM ranged from 0.49 mM to 7.62 mM; isoleucine was 0.76 mM. The medium was sterilized by membrane filtration (Millex-HA, pore size, 0.45 μm; Nihon Millipore, Yonezawa, Japan) and distributed in 10 ml amounts in test tubes (15 × 160 mm) flushed with oxygen-free gas (H_2 10%, CO_2 10%, N_2 80%). The tubes were then stoppered with rubber stoppers. Modified brain-heart infusion (m-BHI) (20) was used as a complex medium. All media were pre-reduced for at least 48 h before use.

Inoculation and incubation

Ten ml of G(–)CDMM were inoculated with 0.1 ml of 10-fold diluted 8 h culture in liver broth and incubated at 37 °C. After 15 h of incubation, 0.1 ml of the culture was inoculated into test media. The inoculated media were then incubated at 37 °C. Inoculation, dilution and incubation were performed under anaerobic conditions. Duplicate culture was performed for each test and mean values are presented in the Results-section. Culture supernates were sterilized by membrane filtration (Millex-HV, pore size, 0.45 μm; Nihon Millipore, Yonezawa, Japan) and used for toxin assays and amino acid analysis.

Bacterial growth

Bacterial growth was determined by measuring the optical density at 560 nm (OD_{560}) of cultures with a Spectronic 20A spectrophotometer (Shimadzu, Kyoto, Japan). The

OD_{560} was measured every 2 h until maximum growth had been reached, then every 24 h over an incubation period of 5 days.

Toxin assay

Toxin A was measured using a direct sandwich enzyme-linked immunosorbent assay (ELISA) (23). The minimum measurable concentration of toxin A in this assay was 10 ng/ml. Toxin B titres were determined using a conventional microtitration plate assay against baby hamster kidney cells (18). The reciprocal of the highest dilution resulting in 100% cell rounding after incubation for 24 h was used as the number of cytotoxic units (CU) per 50 µl of sample (CU/50 µl).

For the assay of intracellular toxins, 10 ml of a culture were centrifuged and the cells were washed twice with 15 mM phosphate-buffered saline, pH 7.2. The cells were suspended in 20 ml of the buffer and sonicated for 6 min with an ultrasonic vibrator (model UR-200P, 20 kilocycles; Tomy Seiko, Tokyo, Japan). The bacterial debris was removed by centrifugation at 12000 *g* for 10 min, then the supernate was filtered through a Millex-HV membrane and assayed for toxins.

Amino acid analysis

Amino acid analysis was carried out on an automatic amino acid analyzer (model JCL-300; JEOL, Tokyo, Japan). The pH values of samples were adjusted to 2.2 with 2 M HCl, and 0.1 ml of the resulting solution was applied to the analyzer.

Results

Determination of amino acids which effected high levels of toxin production

In the basal medium, G(−)CDMM, toxin production was extremely poor: toxin A was not detected (< 10 ng/ml) and the titre of toxin B was 2^2 CU/50 µl. To identify amino acids which effected high levels of toxin production, experiments were performed using G(−)CDMM with the concentration of single amino acids increased up to 100 mM and measuring the toxicity in 5-day culture supernates. Because of low solubility, the concentration of tryptophan was increased to 50 mM. When the concentration of histidine, methionine, valine, isoleucine, proline and leucine was increased, toxin production increased more than 6.9-fold for toxin A and more than 32-fold for toxin B in comparison with those in the basal medium; the toxin A titre in the basal medium was estimated at 10 ng/ml in calculating the factor of increase (Table 1). There was a remarkable increase of toxin production in the presence of 100 mM isoleucine; titres of toxins A and B were 1700 ng/ml and 2^{12} CU/50 µl, respectively, the same as those in m-BHI, although bacterial growth was extremely poor.

Compared with the maximum OD_{560} value (0.22) in G(−)CDMM, bacterial growth was suppressed in glycine, arginine, isoleucine and cysteine (maximum OD_{560} values, 0.11–0.13), and increased in threonine (maximum OD_{560} value, 0.38), when their concentrations were increased.

On the basis of the findings mentioned above, the effect of isoleucine on toxin production was further analyzed in the following experiments.

Table 1. Effects of reinforcement of amino acids on toxin production by *C. difficile* VPI 10463

Basal medium	Amino acid reinforced[1]	Toxin A (ng/ml)	Toxin B [$\log_2$ (CU/50 µl)]
G(−)CDMM	Histidine	110	8
	Tryptophan	38	4
	Glycine	15	4
	Arginine	< 10	2
	Methionine	87	7
	Threonine	23	2
	Valine	870	11.5
	Isoleucine	1700	12
	Proline	69	7
	Leucine	840	11.5
	Cysteine	< 10	–[2]
	None	< 10	2
m-BHI	None	2800	12

[1] Individual amino acids were reinforced at a final concentration of 100 mM except for tryptophan (50 mM).
[2] No cytotoxicity.

Concentration of isoleucine and toxin production

Bacterial growth and toxin production were examined at concentrations of 1, 10, 20, 40, 60, 80, 100 and 200 mM isoleucine. The toxicity of culture supernates was examined on 5 days. Maximum bacterial growth was observed after 10 h incubation at isoleucine concentrations of 1 and 10 mM, and for 12 to 24 h at ≥ 20 mM isoleucine, with the incubation period becoming prolonged with the increasing isoleucine concentrations. The maximum OD_{560} value during incubation was highest (0.27) at 1 mM isoleucine and decreased markedly to 0.15 at 10 mM. Then, gradually, with the increasing isoleucine concentrations of ≥ 20 mM the value decreased to 0.04 at a concentration of 200 mM (Fig. 1). On the other hand, toxin production was extremely poor at 1 and 10 mM, and increased remarkably with the increasing isoleucine concentrations from 20 to 100 mM. The titres of toxins A and B at 1 mM as well as 10 mM were < 10 ng/ml and 2^2 CU/50 µl, respectively. Those at 100 mM isoleucine, at which the maximum OD_{560} value was 0.09, *c.* one-third the value of that at 1 mM, were 1300 ng/ml and 2^{11} CU/50 µl, respectively. This represented more than a 130-fold increase for toxin A and a 512-fold increase for toxin B as compared to the values at 1 mM isoleucine. When the concentration of isoleucine was increased up to 200 mM, toxin production did not increase any further.

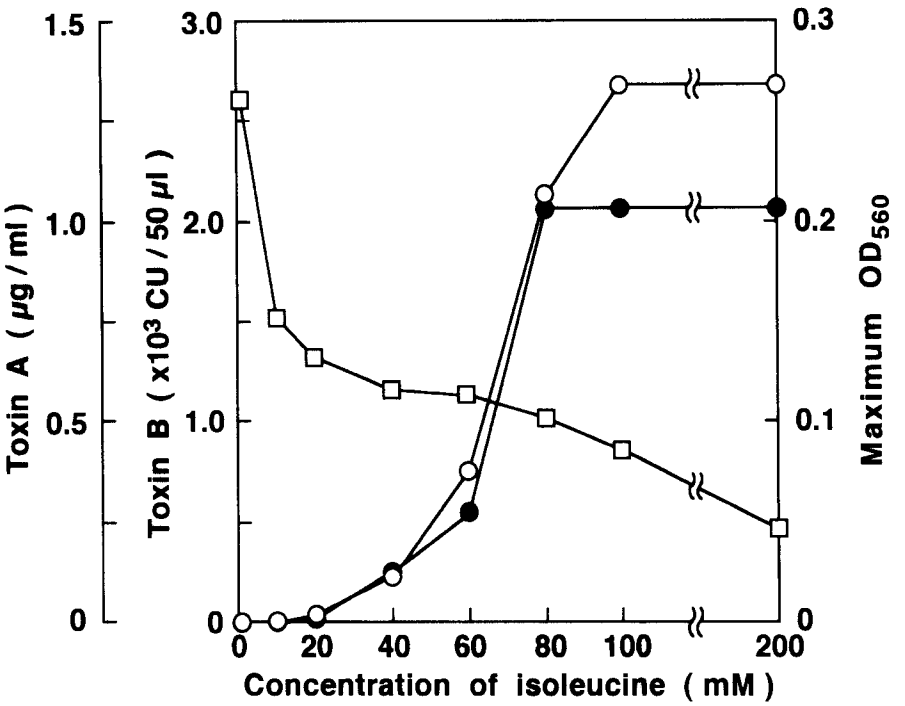


Fig. 1. Toxin production by and bacterial growth of *C. difficile* VPI 10463 at various concentrations of isoleucine. ○, toxin A; ●, toxin B; □, the maximum OD_{560} value during incubation period of 5 days.

In order to examine whether, when isoleucine concentration was increased, its consumption was also increased, consumption of amino acids in the cultures at the concentrations of 1 and 100 mM isoleucine was measured after incubation for 5 days by determining residual concentration of amino acids. There was a distinctive increase in the isoleucine consumption at 100 mM isoleucine in comparison to that at 1 mM; amounts in 10 ml culture consumed at 1 and 100 mM were 10.00 and 290.41 μmol, respectively (Table 2). As to other amino acids, at 100 mM isoleucine consumption of valine, methionine, leucine and proline were clearly decreased and two amino acids, alanine and cystathionine, which were neither contained in the medium nor detected at 1 mM isoleucine, were found at concentrations of 1.54 and 3.54 mM, respectively. Cysteine was completely utilized at both 1 and 100 mM isoleucine.

Incubation period and toxin production

The relationship between incubation period and toxin production was examined at the concentration of 100 mM isoleucine. The amounts of toxins A and

Table 2. Amino acid consumption in 10 ml culture at concentrations of 1 and 100 mM isoleucine after 5 days of incubation

Initial concentration of isoleucine in the medium (mM)	Amino acid consumption[1] (μmol)										
	Thr (8.39)[2]	Gly (13.32)	Val (8.54)	Cys (41.27)	Met (6.70)	Ile	Leu (76.23)	His (6.44)	Trp (4.90)	Arg (5.68)	Pro (69.49)
1 (10)[2]	8.39	1.41	8.54	41.27	6.70	10.00	76.23	0.74	0.31	0.35	27.39
100 (1000)	8.39	1.54	0.86	41.27	2.56	290.41	70.01	0.60	0.16	0.36	19.02

[1] Amount of amino acid in uninoculated medium minus that in 5-day-incubation culture supernate.
[2] Figures in parenthesis indicate the initial amount in 10 ml (μmol).

B were measured in cultures harvested 6, 12, 18, 24, 36, 48, 72, 96 h after inoculation. Both toxins A and B appeared first (toxin A, 26 ng/ml; toxin B, 2^2 CU/50 µl) after 18 h of incubation, at which time bacterial growth was still increasing. Rapid increase in toxin production was observed after incubation for 24 h, and maximum values (toxin A, 1200 ng/ml; toxin B, 2^{11} CU/50 µl) were reached after incubation for 48 h, when bacterial growth was declining. Bacterial growth reached its maximum level (0.11) after incubation for 26 h (Fig. 2). Toxin titres of sonicated cell extracts were consistently quite low throughout incubation; < 10 ng/ml for toxin A and 2^0 to 2^2 CU/50 µl for toxin B.

Toxin titres at 1 mM isoleucine were consistently extremely low, not only in culture supernates, but also in sonicated cell extracts throughout incubation; the maximum values of toxins A and B in culture supernates were 34 ng/ml and 2^4 CU/50 µl, respectively. Those in sonicated cell extracts were < 10 ng/ml and 2^2 CU/50 µl, respectively.

To identify amino acids specifically consumed during toxin production, residual amino acids were analyzed in the same cultures harvested for measurement of the toxins. Before incubation for 24 h, when bacterial growth was in-

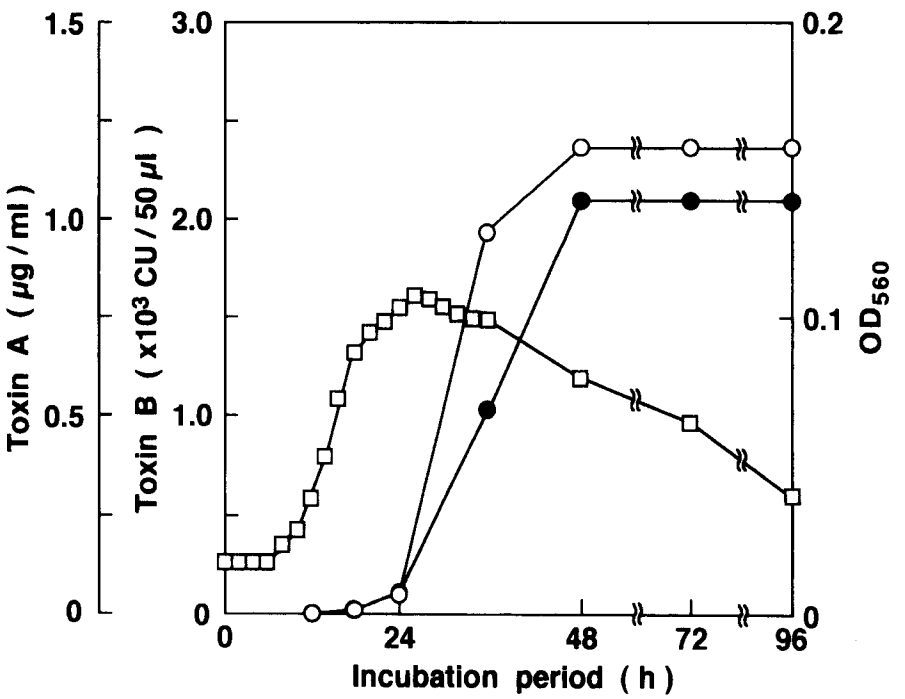


Fig. 2. Time course of toxin production by and growth of *C. difficile* VPI 10463 at 100 mM isoleucine. ○, toxin A; ●, toxin B; □, OD_{560}.

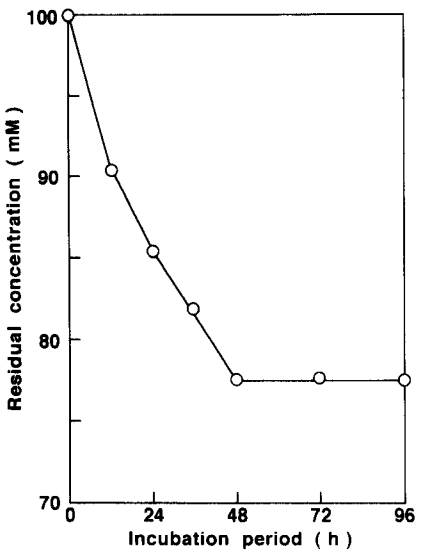
creasing but toxin production remained quite low, all kinds of amino acids except for glycine were taken up, although the amounts taken up varied among the amino acids (Fig. 3). During incubation periods of between 24 and 48 h, when toxin titres increased markedly but bacterial growth was declining, isoleucine, leucine and cysteine were taken up preferentially (Fig. 3a and b). Furthermore, it was noticed that alanine and cystathionine were produced in large quantities during this period (Fig. 3b).

When threonine was completely consumed within 24 h of incubation, the level of toxin titres began to increase (Fig. 3c), suggesting that the complete consumption of threonine specifically led to the toxin production. Therefore, toxin production in G(−)CDMM containing 100 mM isoleucine with 0 or 100 mM threonine was examined. In both media, the same amounts of toxins as those at the original concentration of threonine were produced. In addition, when residual threonine concentration in 96 h culture was analyzed, 1.07 mM remained in the case of G(−)CDMM containing 100 mM threonine.

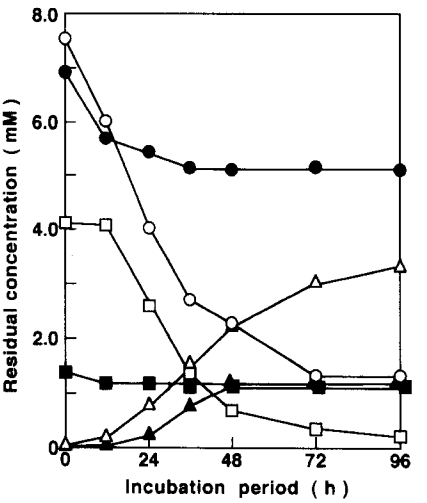
Discussion

C. difficile has an asaccharolytic nature (25) and can produce a large amount of toxins in the gut where there is only a small amount of free carbohydrates (24). This prompted us to analyze nutrients which effected toxin production in the absence of fermentable carbohydrates. In the present study, amino acids which effected toxin production in the absence of fermentable carbohydrates were examined using a minimal medium (13) with a well known highly toxigenic strain VPI 10463. When histidine, methionine, valine, isoleucine, proline or leucine, and particularly isoleucine, were supplemented at high concentration, toxin production showed a distinctive increase, although bacterial growth was not increased in measuring OD_{560}, suggesting that these amino acids at high concentrations were specifically effective for toxin production. *Seddon* et al. (27) examined the effect of arginine and proline on toxin production using the defined medium without fermentable carbohydrates developed by them (25) and found that the addition of proline, but not arginine, showed a small increase in toxin A production, consistent with our results. *Yamakawa* et al. (30) showed that, in the presence of glucose, toxin production was increased, when concentrations of tryptophan, methionine, valine, isoleucine, proline and leucine were increased all together. While they did not examine the effect of each amino acid supplementation, it is quite probable that isoleucine is the most effective promotor of toxin production in the presence of fermentable carbohydrates, as well as in the absence of them, because no growth enhancement was observed at high concentrations of isoleucine; consequently isoleucine does not appear to be important for generation of energy.

Since isoleucine was the most effective promotor of toxin production, its effect was further examined. When the concentration of isoleucine was increased, toxin production was increased, while bacterial growth was sup-



3a



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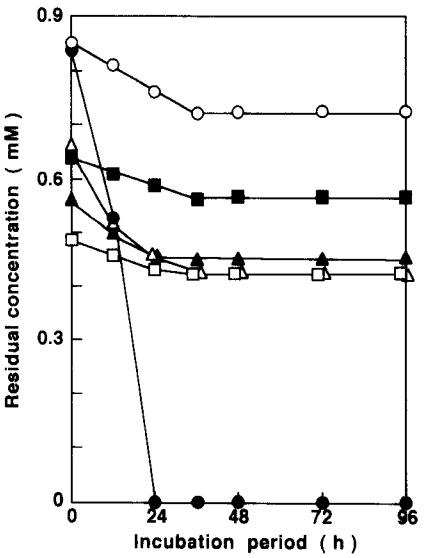

3c

Fig. 3. Time course of amino acid utilization by *C. difficile* VPI 10463 at 100 mM isoleucine. (a) Isoleucine. (b) ○, leucine; ●, proline; □, cysteine; ■, glycine; △, cystathionine; ▲, alanine. (c) ○, valine; ●, threonine; □, tryptophan; ■, histidine; △, methionine; ▲, arginine.

pressed. Considering that titres of toxins A and B in sonicated cell extracts were low at both 1 and 100 mM as compared to those in culture supernates, it seems that the enhancement of toxin production in isoleucine-rich conditions resulted from an enhancement of toxin synthesis rather than toxin release from the cells, as seen in the case of biotin insufficiency (31). Toxin production by *C. difficile* is stimulated by exposure to subinhibitory concentrations of antibiotics, high incubation temperatures, high oxidation-reduction potential and biotin insufficiency (9, 19, 21, 31), suggesting that some stresses on the growth may stimulate toxin production. From this standpoint, a high concentration of isoleucine seems to be one of the important stresses leading to the enhancement of toxin production. The importance of isoleucine in toxin production also seems to be supported by the facts that, at 100 mM isoleucine, consumption of isoleucine was distinctively increased,.and during the incubation period when toxin titres increased markedly but bacterial growth was declining, isoleucine was taken up in a large amount. Recent studies have revealed that the genes encoding toxin A and toxin B form a genetic unit of 19.6 kb together with the three accessory genes, *tcdD (txe1), tcdE (txe2)* and *tcdC (txe3)* (3, 6). More recent works made it manifest that the toxin production was regulated at a transcriptional level (7) and that *tcdD* and *tcdC* have regulatory functions for transcription of the toxin genes (10, 17). It is possible that products in metabolic pathways of isoleucine and related pathways affected by a high concentration of isoleucine may play a role in the triggering of these regulatory genes.

It has been reported that isoleucine has an important role in regulating erythrogenic toxin C production by *Streptococcus pyogenes* (22). In this case, the regulation is explained to be due to the fact that the toxin contains isoleucine in an unusually high concentration in the signal peptide (5). In the case of *C. difficile*, the mechanism of the effect of isoleucine on toxin production seems to be different from that in *S. pyogenes* since toxins A and B do not contain isoleucine in high concentrations (1, 15).

During the incubation period when the level of toxins was increasing markedly and bacterial growth was declining, leucine and cysteine, as well as isoleucine, were taken up preferentially. Considering that cysteine being a reducing agent was utilized completely, even at 1 mM isoleucine, leucine also seems to play an important role in toxin production.

It has been reported that toxin production by *C. difficile* is enhanced in association with production of hydrolytic enzymes such as chondroitin-4-sulphatase, hyaluronate lyase, collagenase, trypsin and subtilisin, when proteose peptone was added to the defined medium (26), suggesting that production of these toxins and hydrolytic enzymes are co-regulated. It is interesting that alanine and cystathionine were produced extracellularly in association with toxin production.

The present study may lead to new insights into the regulation of toxin production by *C. difficile*.

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