Enhancement of *Clostridium difficile* toxin production in biotin-limited conditions

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The effect of biotin on toxin production by *Clostridium difficile* was examined in a defined medium. When toxin production by strain KZ 1647, which was isolated from a healthy adult, was examined in relation to its biotin requirement, it was found that with decreasing concentrations of biotin, bacterial growth was decreased, but production of both toxins A and B were remarkably increased, particularly with 0.05 nM biotin. The time course of production of both toxins in biotin-limited conditions was similar to that in biotin-enriched conditions. The biotin effect on toxin production was also observed in 15 other strains, suggesting that the effect occurs frequently amongst toxigenic *C. difficile* strains. The biotin effect is discussed in relation to the pathogenesis of *C. difficile* colitis.

Introduction

Clostridium difficile causes pseudomembranous colitis and antibiotic-associated diarrhoea [1–4]. Toxins A and B produced by the bacterium are major pathogenic factors [4–6], and although these toxins are now well characterised, the nutritional requirements for toxin production are still unclear. Haslam *et al.* [7] investigated the effect of amino acids on toxin production by *C. difficile* in a defined medium. On the basis of their findings, we recently designed a defined medium composed of a limited number of amino acids, in which *C. difficile* produced both toxins in fairly high amounts [8]. In the course of a study of vitamin requirements for toxin production in this medium, a unique phenomenon was encountered, such that the toxins were produced in much higher amounts in a limited supply of biotin. This paper describes the effect of biotin on toxin production by *C. difficile*.

Materials and methods

Bacterial strains

Sixteen toxigenic *C. difficile* strains were used. These strains were isolated in this laboratory from healthy adults or patients with antibiotic-associated diarrhoea [9]. Strain KZ 1647 from a healthy adult was used in most studies for the examination of the effect of biotin on toxin production.

Preparation of defined medium

A defined medium (6 × MADM) [8], was used with some modifications (Table 1): thiamine, nicotinamide, riboflavin, aminobenzoic acid, folic acid and vitamin B₁₂ were omitted as it was found that these vitamins had no effect on growth [10]. The original concentration of biotin was 50 nM (12.2 µg/L) in this defined medium. The medium was sterilised 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 (H₂ 10%, CO₂ 10%, N₂ 80%). The tubes were then stoppered with rubber stoppers [8].

Inoculation and incubation

Ten ml of the defined medium containing 50 nM biotin were inoculated with 0.1 ml of liver-broth culture that had been incubated for 24 h at 37°C, and were then incubated for 16 h at 37°C. The culture was diluted 1000-fold in pre-reduced saline and 0.1 ml of the diluted culture was inoculated into test media. The carry-over of biotin into the test medium was < 0.0005 nM. The inoculated media were incubated at 37°C in anaerobic conditions (H₂ 10%, CO₂ 10%, N₂ 80%). Duplicate cultures were performed for each test and mean values of the duplicate tests are presented in the *Results* in all cases. Culture supernates sterilised through a membrane (Millex-HV, pore size, 0.45 µm; Nihon Millipore) were used for the toxin assays.

Bacterial growth

Bacterial growth was determined by measuring the

Table 1. Composition of defined medium for *C. difficile*

Component	Concentration	Component	Concentration
Amino acids	(g/L)	Vitamins	(μg/L)
Tryptophan	0.6	Ca-D-pantothenate	1000
Methionine	1.2	Pyridoxine	1000
Valine	1.8	Biotin	12.2
Isoleucine	1.8	Minerals	(mg/L)
Proline	1.8	KH ₂ PO ₄	900
Leucine	2.4	Na ₂ HPO ₄	5000
Cysteine	0.5	NaCl	900
Glycine	0.2	CaCl ₂ ·2H ₂ O	26
Threonine	0.4	MgCl ₂ ·6H ₂ O	20
Glucose	2.0	MnCl ₂ ·4H ₂ O	10
		(NH ₄) ₂ SO ₄	40
		FeSO ₄ ·7H ₂ O	4
		CoCl ₂ ·6H ₂ O	1
		NaHCO ₃	5000

optical density of cultures at 560 nm with a Spectronic 20A spectrophotometer (Shimadzu, Kyoto, Japan).

Toxin assay

Toxin A concentration was determined by the direct sandwich enzyme-linked immunosorbent assay (ELISA) [11]. Male Japanese white rabbits were immunised with highly purified toxin A from *C. difficile* strain VPI 10463 as described by Kamiya *et al.* [12]. IgG antibody to toxin A was purified from immune serum by BioRad Protein MAPS II (BioRad Laboratories, Richmond, CA, USA) and used in the ELISA. The minimum concentration of toxin A measurable in this assay was 10 ng/ml. Toxin B activity was determined by a conventional microtitration plate assay against baby hamster kidney cells [9]. After two-fold serial dilution of culture filtrates, 50 μl of each diluted sample were added to the cells. 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)/50 μl of sample.

For the assay of intracellular toxins, 10 ml of a culture were centrifuged and the cells were washed twice with phosphate-buffered saline, pH 7.2. The cells were suspended in buffer to the original volume and sonicated for 5 min with a Tomy ultrasonic vibrator (mode UR-200P, 20 kilocycles; Tomy Seiko, Tokyo, Japan). The bacterial debris was removed by centrifugation at 12 000 *g* for 10 min, and the supernate was filtered through a Millex-HV membrane and assayed for the toxins.

Results

Concentration of biotin and toxin production

Bacterial growth and toxin production were examined with *C. difficile* strain KZ 1647 at concentrations of 0.00005, 0.0005, 0.005, 0.05, 0.5, 5 and 50 nM biotin. Bacterial growth was measured over an incubation period of 5 days and the toxicity in culture supernates was measured at 5 days. Bacterial growth was maximal

after incubation for 1.5 days at biotin concentrations of 50, 5 and 0.5 nM and for 2 days at concentrations of ≤ 0.05 nM (Fig. 1). The maximum OD₅₆₀ value during incubation was highest (0.48) at a concentration of 50 nM biotin and decreased with the decreasing biotin concentrations; the maximum OD₅₆₀ was < 0.1 at concentrations of ≤ 0.005 nM. On the other hand, toxin production was remarkably increased, when the concentration of biotin was reduced to 0.05 nM (one-thousandth of the original concentration) (Fig. 2), when the maximum OD₅₆₀ value was 0.17, *c.* one-third of that at 50 nM. Toxin production increased 35-fold for toxin A and 64-fold for toxin B; the amounts of toxin A at 50 nM and 0.05 nM biotin were 54 ng/ml and 1880 ng/ml, respectively, and the toxin B activities were 512 CU/50 μl and 32 768 CU/50 μl, respectively. When the concentration of biotin decreased from 0.05 to 0.005 nM, the toxin production markedly decreased, but even at 0.00005 nM biotin, toxin production (toxin

Fig. 1. Bacterial growth of *C. difficile* KZ 1647 at various concentrations of biotin. Biotin concentration: ●, 50 nM; ▲, 0.5 nM; ■, 0.05 nM; ◆, 0.005 nM; ▼, 0.00005 nM. Growth curves at 5 and 0.00005 nM biotin are omitted from the figure to avoid complexity. Each point represents the mean value of duplicate tests.

Fig. 2. Toxin production of *C. difficile* KZ 1647 at various concentrations of biotin. ○, toxin A; ●, toxin B.

A, 495 ng/ml; toxin B, 8192 CU/50 µl) was much higher than that at 50 nM.

Incubation period and toxin production

The relationship between incubation period and toxin production was examined with strain KZ 1647 at the concentrations of 0.05 and 50 nM biotin. The amounts of toxins A and B were measured in cultures harvested 1–5 days after inoculation. The pattern of toxin production at 0.05 nM biotin was similar to that at 50 nM biotin. At either 0.05 or 50 nM biotin, neither toxin was detected at 1 day, both appeared in 2 days, and reached maximum values after incubation for 4 days (Fig. 3). During the incubation period when the toxin production was increasing, bacterial growth was declining as seen in Fig. 1.

Titres of toxins A and B in sonicated cell extracts were consistently low during incubation with either 0.05 or 50 nM biotin: the maximum values of toxins A and B at 0.05 nM biotin were 170 ng/ml (3 days) and 2048 CU/50 µl (3 days), respectively, and those values at 50 nM biotin were 14 ng/ml (3 days) and 128 CU/50 µl (3 days), respectively.

Toxin production by other *C. difficile* strains in biotin-limited defined medium

Toxin production in the defined medium with 0.05 nM biotin after incubation for 5 days was tested for another 15 strains in comparison to that in the defined medium with 50 nM biotin. All 15 strains produced much more toxin A at 0.05 nM biotin than at 50 nM biotin (Fig. 4). Toxin A production increased 2–63-fold (average, 21-fold). The increase in toxin B production was comparable to that of toxin A.

Fig. 3. Time course of toxin production by *C. difficile* KZ 1647 at biotin concentrations of 0.05 nM (○, ●) and 50 nM (□, ■). ○, □, toxin A; ●, ■, toxin B. Each point represents the mean value of duplicate tests.

Discussion

Nutrients affect the toxin production by pathogenic bacteria [13]. In *C. difficile*, lack of essential amino acids suppresses both bacterial growth and toxin production [7,8]. The effect of biotin on toxin production shown in this study is unique in contrast

Fig. 4. Toxin A production by 15 *C. difficile* strains grown with 0.05 nM biotin compared to that at 50 nM biotin. **C. difficile* KZ 1647. Each point represents mean value of duplicate tests.

to the effect of amino acids mentioned above. When the concentration of biotin, which is essential for growth [10], was reduced, growth decreased as expected, whilst toxin production greatly increased, particularly at 0.05 nM biotin, one-thousandth of the optimal concentration for growth. Considering that titres of toxins A and B in sonicated cell extracts were low at either 0.05 or 50 nM, compared with those in culture supernates, it seems that the enhancement of toxin production in the biotin-limited conditions results from an enhancement of toxin synthesis rather than of toxin release from the cells. Cytotoxin (toxin B) production by *C. difficile* in an ordinary complex culture medium is also suggested to be associated not with the release of the toxin during cell lysis but with *de novo* synthesis [14].

Toxin production by *C. difficile* is stimulated by exposure to subinhibitory concentrations of antibiotics, higher incubation temperatures and high oxidation-reduction potential [15–18], suggesting that some stresses on the growth stimulate toxin production. From this standpoint, biotin insufficiency seems to be one of the important stresses leading to the enhancement of toxin production. Furthermore, it is possible that intermediates in the metabolic pathways involving biotin-dependent enzymes may play an important role in the expression of toxin genes, as biotin is a prosthetic group of certain carboxylation-catalysing enzymes [19]. Genes encoding toxins A and B have been cloned [20, 21], yet the molecular basis of gene expression is still unknown. The study of the mechanism of the biotin effect is now in progress in this laboratory.

The present study also showed that the biotin effect occurred in all strains tested, suggesting that it is a general phenomenon amongst toxigenic strains. Therefore, considering that biotin is synthesised by members of the bacterial flora in the human intestine [22, 23], we postulate that the biotin effect may play an important role in the development of colitis. When antibiotics disturb the normal flora, biotin-limited conditions may be produced in the intestine that accelerate toxin production by *C. difficile* and cause colitis. This may partly explain the efficacy of bacteriotherapy for chronic relapsing *C. difficile* diarrhoea [24]. Furthermore, we expect that direct 'biotin treatment' will open a new window to control the disease from both a prophylactic and therapeutic aspect. In this respect it might be necessary to examine the amount of biotin in the faeces of patients with *C. difficile* colitis. It would also be of interest to determine whether the biotin effect occurs in other toxigenic bacteria that cause intestinal infection.

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