BACTERIAL PATHOGENICITY AND CHARACTERISATION

Inhibition of enhanced toxin production by *Clostridium difficile* in biotin-limited conditions

K. YAMAKAWA, T. KARASAWA, T. OHTA*, H. HAYASHI† and S. NAKAMURA

Department of Bacteriology, School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8640,

*College of Medical Technology and Nursing and †Department of Microbiology, Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

Production of toxins A and B by *Clostridium difficile* is enhanced in a defined medium with biotin-limited conditions. In the present study compounds inhibitory to enhanced toxin production by a *C. difficile* strain were examined. Increases in biotin concentration from 0.05 nM to 50 nM accelerated growth and inhibited enhanced toxin production. Asparagine, glutamic acid and glutamine (10 mM) showed an effect on growth and toxin production similar to that of biotin. Lysine (10 mM) suppressed growth and inhibited toxin production. Addition of these toxin-inhibitory compounds within an incubation period of 2 days inhibited the enhanced toxin production, but later addition showed only slight inhibition of toxin production. Amino acids contained in the defined medium under the biotin-limited conditions were actively utilised in the presence of the three toxin-inhibitory amino acids, but in the presence of lysine, amino-acid utilisation was suppressed. Different mechanisms of action of these toxin-inhibitory molecules, which may be divided into excess biotin, asparagine-glutamic acid-glutamine group, and lysine, are discussed.

Introduction

Clostridium difficile is the pathogen responsible for most cases of pseudomembranous colitis and many cases of antibiotic-associated diarrhoea. The bacterium produces toxins A and B [1–5]. An earlier study showed that the production of these toxins by *C. difficile* is enhanced in a biotin-limited defined medium (biotin effect) and it was hypothesised that administration of excess biotin to a patient with *C. difficile* colitis may have a therapeutic effect [6].

Enzymes containing biotin as a prosthetic group play significant roles in various physiological pathways, such as the metabolism of glucose, amino acids, fatty acids and nucleic acids [7]. As overall deterioration in cell physiology due to biotin insufficiency may be related to enhanced toxin production, it is difficult to determine the direct contribution of biotin to toxin regulation.

Nine amino acids – tryptophan, methionine, valine, isoleucine, proline, leucine, cysteine, glycine and threonine [8] – from the 18 amino acids in the defined medium of Haslam *et al.* [9] were selected for

the development of a defined medium to achieve better toxin production. The first seven amino acids are essential for bacterial growth and the last two are growth-enhancing [6, 10]. Toxin production by *C. difficile* was higher in this defined medium than in Haslam's medium. Therefore, it was likely that an omitted amino acid(s) suppresses toxin production by *C. difficile*.

The present study examined the inhibitory effect of certain amino acids, as well as excess biotin, on enhanced toxin production by *C. difficile* in a biotin-limited defined medium and analysed changes in amino-acid consumption in media with and without inhibitory amino acids.

Materials and methods

Bacterial strain

A toxigenic *C. difficile* strain KZ 1647 was used. This strain showed a typical response of enhanced toxin production in a biotin-limited defined medium [6].

Defined medium and cultivation

A biotin-limited defined medium [6] containing biotin (0.05 nM) and the following amino acids, tryptophan,

methionine, valine, isoleucine, proline, leucine, cysteine, glycine and threonine, was used. The medium was inoculated for toxin production as described previously [6] and incubated anaerobically at 37°C for 5 days. To evaluate the effect of a test compound, 0.2 ml of the concentrated solution (pH 7.4) was added to a culture medium (10 ml) while flushing with oxygen-free gas. The same volume of saline was added to other tubes of culture medium as a negative control.

Bacterial growth and toxin assay

Bacterial growth was determined by measuring the optical density of cultures at 560 nm as described previously [6]. Levels of toxins A and B in culture supernates and in sonicated cell extracts were determined by an enzyme-linked immunosorbent assay and by a conventional cytotoxicity assay with baby hamster kidney cells, respectively, as reported previously [6]. Experiments were conducted in duplicate and mean values are presented.

Amino acids analysis

Amino acid concentrations in membrane-filtered (Milliplex GV, Millipore, Yonezawa, Japan) cultures were determined with a JLC-300 amino acid analyser (JEOL, Tokyo, Japan).

Results

Inhibition of *C. difficile* toxin production with excess biotin

Biotin (50 nM) was added to cultures of strain KZ 1647 at different incubation periods and then bacterial growth and toxin production were determined. Excess biotin increased bacterial growth within 1 day of its addition to the cultures (Fig. 1). Addition of biotin at early incubation times yielded a remarkable response in growth. Furthermore, a growth increase was still shown by addition to 4-day-old cultures. Toxin production was clearly inhibited by the addition of biotin to 1- and 2-day-old cultures; toxin A levels did not exceed 200 ng/ml, about one-tenth of the level (1880 ng/ml) achieved in biotin-limited conditions (Fig. 2). However, in the cultures to which biotin was added on the third day of incubation or later, the toxin level was similar to that produced in biotin-limited conditions. Toxin B levels paralleled those of toxin A.

Inhibition of toxin production by amino acids

Toxin production by strain KZ 1647 was examined in the biotin-limited defined medium supplemented with nine amino acids (g/L): alanine (0.2), arginine (0.2), aspartic acid (0.3), glutamic acid (0.9), histidine (0.1), lysine (0.3), phenylalanine (0.2), serine (0.3) and tyrosine (0.1) (concentration of supplemented amino acid was the same as that in complete amino acid-

Fig. 1. Effect of biotin increase or addition of glutamine and lysine on growth of *C. difficile* KZ 1647. a, 50 nM biotin; b, 10 mM glutamine; c, 10 mM lysine. Timing of addition is indicated by arrows. Dotted base line is a growth curve in biotin-limited conditions.

defined medium [9]). Toxin levels were low compared to those in biotin-limited defined medium without the supplementation. The levels of toxins A and B were 2050 ng/ml and $2^{14.0}$ CU/50 μ l in the non-supplemented medium and <10 ng/ml and $2^{3.5}$ CU/50 μ l, respectively, in the supplemented medium.

On the basis of these findings, single amino acids used in the above supplementation experiment were added to the medium at 10 mM, and toxin production was determined to identify amino acid(s) inhibitory to the enhanced toxin production. An inhibitory effect was observed with lysine, asparagine, glutamic acid and glutamine (Table 1); the levels of toxins A and B were ≤ 85 ng/ml and $\leq 2^{3.0}$ CU/50 μ l, respectively. Intracellular toxin titres in the cultures with inhibitory amino acids were low; levels of toxins A and B were <10 ng/ml and 2 CU/50 μ l, respectively, in the cultures with asparagine, glutamic acid or glutamine,

Fig. 2. Inhibition of enhanced toxin A production by *C. difficile* KZ 1647 by biotin increase or addition of inhibitory amino acids. **a**, 50 nM biotin; **b**, 10 mM asparagine; **c**, 10 mM glutamic acid; **d**, 10 mM glutamine; **e**, 10 mM lysine. Toxin A titre was determined after incubation for 5 days.

and 12 ng/ml and 2 CU/50 μ l, respectively, in the cultures with lysine. Intracellular toxin A and B levels in the cultures without the inhibitory amino acids were 70 ng/ml and 2⁸ CU/50 μ l, respectively.

To analyse further the inhibitory effect of the four amino acids, they were added after different periods of incubation and then growth and toxin production were determined. When glutamine was added to 1-day-old cultures, bacterial growth increased by about double

Table 1. Inhibitory effect of amino acids on toxin production by *C. difficile* KZ 1647 in a biotin-limited defined medium

Amino acid added (10 mM)*	Toxin A (ng/ml)	Toxin B (log ₂ CU/50 μ l)
None	1940	14.5
Aspartic acid	1810	14.5
Alanine	1270	14.0
Phenylalanine	1220	14.0
Serine	1070	14.0
Arginine	1010	13.5
Histidine	730	12.5
Lysine	85	5.0
Asparagine	38	5.0
Glutamic acid	<10	3.0
Glutamine	<10	3.5

*The basal defined medium was biotin-limited (0.05 nM) and contained nine amino acids: tryptophan, methionine, valine, isoleucine, proline, leucine, cysteine, glycine and threonine.

the maximum growth of cultures without glutamine (Fig. 1). A growth increase still occurred with the addition of glutamine to 2–4-day-old cultures. The addition of glutamic acid or asparagine showed a similar effect on growth that was not as marked as with glutamine (data not shown). However, with lysine, growth was suppressed following addition to 1-day-old cultures and lysine added after incubation for 2 days or later showed no influence on growth (data not shown). The addition of glutamine to 1- or 2-day-old cultures almost completely inhibited toxin A production, while for 3-day cultures glutamine inhibited toxin A to about half the level produced in the cultures without glutamine (Fig. 2). The addition of asparagine, glutamic acid, or lysine to 1- or 2-day-old cultures was also highly inhibitory and toxin B levels in these cultures paralleled those of toxin A.

Amino-acid consumption in the presence of the inhibitory amino acid

Amino-acid concentrations in culture supernates after incubation of strain KZ 1647 for 5 days with and without inhibitory amino acids were determined. The inhibitory amino acids were added at the beginning of incubation in this experiment. In cultures without inhibitory amino acids, threonine was almost totally consumed, as was approximately half of the leucine, isoleucine and proline (Table 2). Valine, methionine, tryptophan and glycine were not significantly utilised. When the inhibitory amino acid was added to the medium, asparagine, glutamic acid, glutamine and lysine were consumed in amounts of 1.14, 1.79, 4.75 and 1.53 μ mol/ml, respectively. Differences in leucine and isoleucine consumption were observed in media containing inhibitory amino acids. Consumption of leucine and isoleucine was increased in media containing asparagine, glutamic acid or glutamine, but consumption in the medium containing lysine was similar to that in the medium without inhibitory amino acids. This feature was observed also for valine and methionine.

Table 2. Concentration of amino acids in the biotin-limited medium after incubation of strain KZ 1647 for 5 days

Amino acid added	Amino-acid concentration (mM)							
	Thr	Leu	Ile	Pro	Val	Met	Trp	Gly
None (uninoculated)	3.36	18.30	13.72	15.63	15.36	8.04	2.94	2.66
None	0.34	8.85	6.21	9.10	15.02	8.13	2.84	2.65
10 mM Asn	0.40	0.00	4.48	8.20	13.44	6.74	2.67	2.59
10 mM Glu	0.03	2.27	4.80	9.62	13.96	6.66	2.75	2.49
10 mM Gln	0.01	2.01	4.25	8.49	13.27	6.80	2.69	2.41
10 mM Lys	0.52	8.79	6.65	8.11	15.07	8.06	2.86	2.62

Mean concentrations from triplicate cultures in each medium are presented. The cysteine concentration has been omitted because an accurate analysis was not performed.

Discussion

As well as excess biotin, four amino acids were shown to be inhibitory to enhanced toxin production by *C. difficile* in biotin-limited conditions. Growth of *C. difficile* KZ 1647 recovered following biotin supplementation. This growth recovery may reflect 'normal' physiology of the bacterial cells, which is related to a certain relaxed state of toxin production. The addition of excess biotin to cultures after incubation for up to 2-days, the early phase of toxin production [6], caused such relaxation. However, biotin added from the third day of incubation did not inhibit enhanced toxin production, even though a considerable increase in vegetative growth was observed. These findings suggest that enhanced toxin production includes an irreversible course of cell physiology. Toxin synthesis by *C. difficile* in a complex medium may be related to early events of sporulation [11]. In the present system, the decline phase of growth of strain KZ 1647 started after incubation for *c.* 2 days and sporulation then occurred (unpublished data). Therefore, it is likely that the irreversible process of enhanced toxin production is linked to a physiological change from vegetative growth to sporulation.

Amino acids regulate neurotoxin production by *C. botulinum* in defined media: excess arginine and tryptophan markedly decrease neurotoxin production by *C. botulinum* type A and B strains, and type E strains, respectively [12, 13]. In the latter case, tryptophan is an essential nitrogen source in a minimal medium. Leyer and Johnson [13] suggested that decreased toxin titres were related specifically to tryptophan metabolism, although growth was not affected by excess tryptophan. They also suggested that toxin repression by tryptophan may indicate nutritional sufficiency of the cell and pointed out the importance of other nitrogen sources in the control of toxin production. Both reports [12, 13] further described a similar effect with glutamic acid to some degree, although glutamic acid did not increase growth.

The defined medium used in the present study contained the minimal number of amino acids for good growth of *C. difficile* [9]. The amino acids inhibitory to toxin production, i.e., asparagine, glutamic acid, glutamine and lysine, have no effect on the

growth of *C. difficile* [9, 10]. However, under biotin-limited conditions, glutamine, glutamic acid and asparagine yielded better growth than the minimal combination of amino acids. Toxin inhibition by glutamic acid was similar to that seen with neurotoxin production by *C. botulinum*, although *C. difficile* growth was increased to some degree. Glutamine, glutamic acid and asparagine play central roles in clostridial physiology such as in amino-acid catabolism [14]. Therefore, availability of these three amino acids may compensate a biased physiology with low functional biotin enzymes.

Lysine biosynthesis is achieved through many enzymic steps in the genus *Clostridium* as with other genera [14]. However, lysine is not essential for growth of *C. difficile* [9, 10], and is growth suppressive under biotin-limited conditions. The inhibition of toxin production by lysine may be different to that caused by asparagine, glutamic acid and glutamine mentioned above.

The molecular mechanisms of *C. difficile* toxin regulation have been studied progressively. Recently, it was shown that transcriptional activation of the toxin A and B genes is induced at the post-exponential growth phase in a complex medium and it was suggested that positive and negative regulation genes control the expression of these toxin genes [15–18]. It will be interesting to learn whether intracellular biotin concentration influences the function or expression of regulation proteins. In any case, knowledge of the physiological background of *C. difficile* cells is necessary for any investigation of *C. difficile* toxigenesis at the molecular level.

The nutritional environment in the human colon (the site of *C. difficile* infection) is different to our simplified model due to the presence of non-digested polysaccharides and mucopolysaccharides with trace amounts of glucose, and an unknown balance of amino acids and polypeptides. Moreover, the intestinal environment is dramatically changed both bacteriologically and nutritionally when antibiotics are administered. For these reasons the mechanism of *C. difficile* toxin production should be further elucidated.

We are grateful to the Chemical Analysis Centre, University of Tsukuba, for amino acid analysis.

References

1. Lierly DM, Krivan HC, Wilkins TD. *Clostridium difficile*: its disease and toxins. *Clin Microbiol Rev* 1988; **1**: 1–18.
2. Dove CH, Wang S-Z, Price SB *et al*. Molecular characterization of the *Clostridium difficile* toxin A gene. *Infect Immun* 1990; **58**: 480–488.
3. Johnson JL, Phelps C, Barroso L, Roberts MD, Lierly DM, Wilkins TD. Cloning and expression of the toxin B gene of *Clostridium difficile*. *Curr Microbiol* 1990; **20**: 397–401.
4. Just I, Wilm M, Selzer J *et al*. The enterotoxin from *Clostridium difficile* (ToxA) monoglucosylates the Rho proteins. *J Biol Chem* 1995; **270**: 13932–13936.
5. Just I, Selzer J, Wilm M, von Eichel-Streiber C, Mann M, Aktories K. Glucosylation of Rho proteins by *Clostridium difficile* toxin B. *Nature* 1995; **375**: 500–503.
6. Yamakawa K, Karasawa T, Ikoma S, Nakamura S. Enhancement of *Clostridium difficile* toxin production in biotin-limited conditions. *J Med Microbiol* 1996; **44**: 111–114.
7. Lehninger AL, Nelson DL, Cox MM. Principles of biochemistry, 2nd edn. New York, Worth Publishers. 1993.
8. Yamakawa K, Kamiya S, Meng XQ, Karasawa T, Nakamura S. Toxin production by *Clostridium difficile* in a defined medium with limited amino acids. *J Med Microbiol* 1994; **41**: 319–323.
9. Haslam SC, Ketley JM, Mitchell TJ, Stephen J, Burdon DW, Candy DCA. Growth of *Clostridium difficile* and production of toxins A and B in complex and defined media. *J Med Microbiol* 1986; **21**: 293–297.
10. Karasawa T, Ikoma S, Yamakawa K, Nakamura S. A defined growth medium for *Clostridium difficile*. *Microbiology* 1995; **141**: 371–375.
11. Kamiya S, Ogura H, Meng XQ, Nakamura S. Correlation between cytotoxin production and sporulation in *Clostridium difficile*. *J Med Microbiol* 1992; **37**: 206–210.
12. Patterson-Curtis SI, Johnson EA. Regulation of neurotoxin and protease formation in *Clostridium botulinum* Okra B and Hall A by arginine. *Appl Environ Microbiol* 1989; **55**: 1544–1548.
13. Leyer GJ, Johnson EA. Repression of toxin production by tryptophan in *Clostridium botulinum* type E. *Arch Microbiol* 1990; **154**: 443–447.
14. Andreesen JR, Bahl H, Gottschalk G. Introduction to the physiology and biochemistry of the genus *Clostridium*. In: Minton NP, Clarke DJ (eds) *Clostridia*. New York, Plenum Press. 1989: 27–62.
15. Braun V, Hundsberger T, Leukel P, Souerborn M, von Eichel-Streiber C. Definition of the single integration site of the pathogenicity locus in *Clostridium difficile*. *Gene* 1996; **181**: 29–38.
16. Hammond GA, Lierly DM, Johnson JL. Transcriptional analysis of the toxigenic element of *Clostridium difficile*. *Microb Pathog* 1997; **22**: 143–154.
17. Hundsberger T, Braun V, Weidmann M, Leukel P, Souerborn M, von Eichel-Streiber C. Transcription analysis of the genes *tcdA–E* of the pathogenicity locus of *Clostridium difficile*. *Eur J Biochem* 1997; **244**: 735–742.
18. Moncrief JS, Barroso LA, Wilkins TD. Positive regulation of *Clostridium difficile* toxins. *Infect Immun* 1997; **65**: 1105–1108.