NEW DIAGNOSTIC METHODS

Toxin production by *Clostridium difficile* in a defined medium with limited amino acids

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Summary. Basal defined medium (BDM) containing vitamins, minerals and seven amino acids—(L) tryptophan 0.1 g, methionine 0.2 g, valine 0.3 g, isoleucine 0.3 g, proline 0.3 g, leucine 0.4 g and cysteine 0.5 g—which appeared to be essential for good growth of *Clostridium difficile* was prepared. Addition of glycine 0.2 g/L and threonine 0.4 g/L to BDM produced better growth of strain VPI 10463, and this defined medium was designated minimum amino acid-defined medium (MADM). Production of toxins A and B by strain VPI 10463 in 6 × MADM containing (L) tryptophan 0.6 g, methionine 1.2 g, valine 1.8 g, isoleucine 1.8 g, proline 1.8 g, leucine 2.4 g, cysteine 0.5 g, glycine 0.2 g and threonine 0.4 g, was much greater than in MADM. Toxin production by 20 *C. difficile* strains was examined in two defined media—6 × MADM and complete amino acid-defined medium (CADM) containing 18 amino acids—and one complex medium, modified brain heart infusion medium (m-BHI). Simultaneous production of toxins A and B by all test strains was demonstrated in m-BHI and the two defined media. It was also shown that 6 × MADM was generally better than CADM and as effective as m-BHI for stimulating toxin production by 13 strains. This defined medium would be useful for studies on the physiology, metabolism and pathogenicity of *C. difficile*.

Introduction

Clostridium difficile causes pseudomembranous colitis and is a major aetiological agent of antibiotic-associated diarrhoea.^{1–4} It produces at least two toxins designated toxin A (enterotoxin) and toxin B (cytotoxin), which are considered to be important virulence factors.^{5–8}

The composition of the growth medium greatly influences the ability of *C. difficile* to produce these toxins *in vitro*.⁹ However, little work has been reported on the nutritional requirements for toxin production. Haslam *et al.*¹⁰ demonstrated that seven amino acids—tryptophan, methionine, valine, isoleucine, proline, leucine and cysteine—are essential for good growth, and that the amino acids required for toxin production varied between strains.

In the present study, toxin production by *C. difficile* in a defined medium containing the amino acids essential or stimulatory for growth was investigated.

Materials and methods

Bacterial strains

Twenty *C. difficile* strains were used in this study. Reference strain VPI 10463 was used to determine the composition and concentration of amino acids required in a defined medium. The other 19 strains had been isolated in our laboratory from healthy adults (13 strains) or patients with antibiotic-associated diarrhoea (six strains),¹¹ and were tested for toxin production in the proposed medium. Toxin B production in m-BHI medium (see below) was in the range 2⁵–2¹⁴ CU/50 µl in the strains from healthy adults and 2⁶–2¹³ CU/50 µl in the strains from symptomatic patients.

Preparation of defined media

Basal defined medium (BDM) (table) was based on that described by Haslam *et al.*¹⁰ with four modifications: Na₂HPO₄ was added, Na₂CO₃ was replaced with NaHCO₃, the concentration of glucose was 0.2% instead of 0.5%, and 11 amino acids—histidine, glycine, tyrosine, arginine, phenylalanine, threonine, alanine, lysine, serine, aspartic acid and glutamic acid—were omitted. The medium was sterilised by membrane filtration (Millex-HA, pore size, 0.45 µm; Nihon Millipore, Yonezawa, Japan) and distributed in

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Table. Composition of basal defined medium (BDM) and complete amino acid-defined medium (CADM)

Amino acids (g)		Vitamins (mg)	
Tryptophan	0.1	Thiamine	1000
Methionine	0.2	Ca-D-pantothenate	1000
Valine	0.3	Nicotinamide	1000
Isoleucine	0.3	Riboflavin	1000
Proline	0.3	Pyridoxine	1000
Leucine	0.4	p-Aminobenzoic acid	50
Cysteine	0.5	Biotin	12.5
Histidine*	0.1	Folic acid	12.5
Glycine*	0.1	B ₁₂	5.0
Tyrosine*	0.1		
Arginine*	0.2	Minerals (mg)	
Phenylalanine*	0.2	KH ₂ PO ₄	900
Threonine*	0.2	Na ₂ HPO ₄	5000
Alanine*	0.2	NaCl	900
Lysine*	0.3	CaCl ₂ ·2H ₂ O	26
Serine*	0.3	MgCl ₂ ·6H ₂ O	20
Aspartic acid*	0.3	MnCl ₂ ·4H ₂ O	10
Glutamic acid*	0.9	(NH ₄) ₂ SO ₄	40
		FeSO ₄ ·7H ₂ O	4
Glucose (g)	2.0	CoCl ₂ ·6H ₂ O	1
		NaHCO ₃	5000
		Distilled water (L)	
		1	

*Amino acids contained in CADM but not in BDM.

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. Complete amino acid-defined medium (CADM) containing all 18 amino acids was also prepared as described above. Modified Brain Heart Infusion Broth (m-BHI) was prepared according to the description by Nakamura *et al.*¹²

Inoculation and incubation

Standard suspensions for inoculation were prepared after growth of *C. difficile* in liver broth for 24 h at 37°C. The cultures were centrifuged at 1500 *g* for 20 min and washed twice with pre-reduced saline. The final pellets were resuspended in the original volumes of saline and used as inocula.

To determine the growth-enhancing amino acids for strain VPI 10463, 0.1 ml of the suspension was inoculated into 10 ml of BDM supplemented with one of the specified amino acids at the concentration shown in the table. The cultures were then incubated at 37°C and bacterial growth was measured every 2 h.

For investigation of toxin production, 10 ml of various test media were inoculated with 0.1 ml of the inoculum suspension and incubated for 16 h at 37°C. The cultures were diluted 1000-fold in pre-reduced saline and 0.1 ml of the diluted culture was used for inoculation of 10-ml volumes of the various test media. Thus, the carry-over of liver broth into a test medium was negligible. The cultures were incubated at 37°C for 5 days in anaerobic conditions and culture supernates were collected by centrifugation. The supernates were sterilised by membrane filtration (Millex-GV, pore

size, 0.22 µm; Nihon Millipore) and used for the toxin assays.

Bacterial growth

Bacterial growth was determined by measuring the optical density of cultures at 560 nm (OD₅₆₀) with a Shimadzu-Bosch-Lomb Spectronic 20A spectrophotometer (Shimadzu, Kyoto, Japan).

Toxin assays

Toxin A was quantified by the direct sandwich enzyme-linked immunosorbent assay (ELISA) as described by Redmond *et al.*¹³ The minimum concentration of toxin A measurable in this assay was 10 ng/ml.

Toxin B titres were determined by a conventional cytotoxicity test with BHK-21/WI-2 cells.¹² After two-fold serial dilution of unconcentrated culture filtrates, each diluted sample was added to BHK-21/WI-2 cells. The cytotoxic titre was the maximum dilution that gave 100% cell rounding after incubation for 24 h.

Results

Determination of growth-enhancing amino acids

Growth of strain VPI 10463 in BDM was considerably poorer than that in CADM; the maximum OD₅₆₀ values were 0.60 in BDM and 0.88 in CADM, and the incubation time required to reach these values was 14 h in BDM and 9 h in CADM.

To identify the growth-enhancing amino acids, growth of strain VPI 10463 in BDM supplemented with one of 11 amino acids, which were contained in CADM but not in BDM, was examined; threonine and glycine enhanced growth considerably. In BDM supplemented with threonine, maximum growth (OD₅₆₀ 0.75) was reached after incubation for 11 h (fig. 1). In BDM supplemented with glycine, significantly increased growth was observed from 4 to 14 h after inoculation in comparison to growth in BDM although the maximum OD₅₆₀ value was not markedly increased. Two-fold greater concentration of these amino acids did not show any further effect.

Toxin production in defined media

BDM and BDM supplemented with threonine 0.4 g/L and glycine 0.2 g/L (minimum amino acid-defined medium; MADM) were compared for toxin production by strain VPI 10463. The amounts of both toxins produced were approximately two-fold more in MADM than in BDM; the amounts of toxins A and B were 440 ng/ml and 2¹³ CU/50 µl in BDM, and 980 ng/ml and 2¹⁴ CU/50 µl in MADM, respectively.

The concentrations of six essential amino acids—tryptophan, methionine, valine, isoleucine, proline and

Fig. 1. Effect of glycine 0.1 g/L (Δ) and threonine 0.2 g/L ($\blacktriangle$) on the growth of *C. difficile* strain VPI 10463 in BDM; $\circ$, no amino acid supplement; $\bullet$, CADM.

Fig. 2. Effect of increments of amino acids on toxin production of toxins A ($\circ$) and B ($\bullet$) by *C. difficile* strain VPI 10463 in MADM. Basal concentration of amino acids (g/L): tryptophan, 0.1; methionine, 0.2; valine, 0.3; isoleucine, 0.3; proline, 0.3; leucine, 0.4; glycine, 0.2; threonine, 0.4; cysteine, 0.5. Concentrations of amino acids except for glycine, threonine and cysteine were increased.

leucine—in MADM were increased up to 10 times those in BDM to determine their relationship with toxin production. The amount of toxin A increased in parallel with the increase in concentrations of these amino acids, reaching a maximum (3360 ng/ml) when five times the basal concentrations of the amino acids were used (fig. 2). In contrast, the amount of toxin B reached a maximum (2^{15} CU/50 μ l) when twice the basal concentrations of the amino acids were used. Bacterial growth did not fluctuate in the range of

concentrations tested; the maximum OD₅₆₀ values were 0.41–0.48.

On the basis of these findings, a defined medium (6 $\times$ MADM) containing (g/L) tryptophan 0.6, methionine 1.2, valine 1.8, isoleucine 1.8, proline 1.8, leucine 2.4, glycine 0.2, threonine 0.4 and cysteine 0.5 was prepared and compared with m-BHI and CADM with respect to toxin production.

All 20 test strains produced detectable amounts of toxin B in any medium, although the titres of the toxin

Fig. 3. Comparison of toxin B production by *C. difficile* strains in 6xMADM to that in m-BHI (○) or CADM (●). Difference of toxin B titres between m-BHI and 6xMADM, or CADM and 6xMADM, for individual strains shown between two dashed lines was less than four-fold.

Fig. 4. Correlation between production of toxins A and B by *C. difficile* strains in m-BHI (○), 6xMADM (●) and CADM (△). The regression equation gave: m-BHI, $Y = 0.23X - 0.01$, $r = 0.99$, $n = 18$; 6xMADM, $Y = 0.22X + 0.12$, $r = 0.98$, $n = 13$; CADM, $Y = 0.21X + 0.02$, $r = 0.98$, $n = 9$.

varied among the three media. As there was a correlation between production of toxins A and B in all three media tested as described below, the three media were evaluated according to the titres of toxin B (fig. 3).

In a comparison of 6xMADM and m-BHI, 13 strains did not produce significantly different amounts of toxin B; differences in the titres between the two

media for individual strains were less than four-fold. In a comparison of 6xMADM and CADM, it was observed generally that 6xMADM was more effective than CADM for stimulating toxin B production. Five strains produced eight times more toxin B in 6xMADM than in CADM, and none of the strains produced eight times more toxin B in CADM than in 6xMADM.

Toxin A was detected with the ELISA employed in this study in all cultures in which the titre of toxin B was $\geq 2^6$ CU/50 μ l. Therefore, correlation between production of toxins A and B in the respective media was analysed for the strains producing toxin B at $\geq 2^6$ CU/50 μ l (fig. 4); 18 strains were used for the analysis in m-BHI, 13 in 6xMADM, and nine in CADM. All the strains produced both toxins in parallel concentrations in defined media (6xMADM and CADM) and in the complex medium (m-BHI). These results indicate that the strains producing toxin B at $< 2^6$ CU/50 μ l may also produce toxin A. When 6xMADM culture supernatant fluids of six strains that produced undetectable amounts of toxin A and 2^5 CU of toxin B/50 μ l were concentrated with polyethylene glycol 20000 (resulting in a toxin B titre of 2^{8-9} CU/50 μ l), 96–190 ng of toxin A/ml were detected.

Discussion

Haslam *et al.*¹⁰ demonstrated that seven amino acids—tryptophan, methionine, valine, isoleucine, proline, leucine and cysteine—were essential for good growth of *C. difficile*. Therefore, a medium composed of these seven amino acids was used as BDM in the studies. Bacterial growth in BDM was enhanced by the addition of glycine or threonine, although the maximum growth was slightly less than that in CADM containing 18 amino acids. It is possible that some amino acids other than glycine and threonine may enhance bacterial growth to a level which is not detected by the methods employed here.

Cysteine is commonly used at a concentration of 0.05% as a reducing agent in anaerobic culture; it inhibits bacterial growth at higher concentrations.¹⁴ Therefore, various concentrations of the six essential amino acids except for cysteine were examined for stimulation of toxin production by *C. difficile*. Maximum production of toxins A and B was observed when five-to-nine times more amino acids were added to BDM. It remains to be determined which amino acid is the most effective for stimulating toxin production by *C. difficile*.

Haslam *et al.*¹⁰ reported that weakly toxigenic strains may require some amino acids for production of toxin A, in addition to those essential for bacterial growth. The strains tested in this study varied widely in toxigenicity as determined in m-BHI; the titre of toxin A ranged between < 10 and 3360 ng/ml and that of toxin B between 2^5 and 2^{16} CU/50 μ l. However, all 20

strains tested produced both toxins A and B in a defined medium, 6 × MADM, composed of seven essential and two amino acids that stimulated growth. Furthermore, five strains produced distinctively larger amounts of toxins in 6 × MADM than in CADM. This may be due to differences between the strains tested at high concentrations of amino acids. Recently, it was reported that the concentration of arginine or tryptophan markedly affects the production of neurotoxin by group I or type E strains of *C. botulinum*, respectively.^{15,16}

In complex media such as m-BHI, toxigenic *C. difficile* strains, with some exceptions, generally produced both toxins A and B in parallel concentrations.¹⁷⁻¹⁹ This correlation was also found when the defined medium, 6 × MADM was used, indicating that amino acids included in this medium are sufficient for the production of both toxins as far as the strains tested are concerned.

Toxins A and B are encoded by two separate genes located in close proximity on the chromosome.^{20,21}

Toxigenic strains with both toxin A and toxin B genes generally produce both toxins simultaneously.^{22,23} This suggests that simultaneous expression of the two toxins is due to the presence of both genes, although the unusual toxin A-negative, toxin B-positive strain was reported recently to have a whole toxin B gene and an incomplete toxin A gene.^{24,25} The correlation between the levels of toxin A and toxin B produced also indicates that expression of both toxin genes may be regulated in the defined medium in the same manner as in the complex medium.

Considering that nearly half of the strains tested produced fairly large amounts of both toxins, the defined medium developed in this study will be useful for studies on the physiology, metabolism and pathogenicity of *C. difficile*, and particularly for the study of various environmental factors affecting the expression of toxin genes.

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