Growth of *Clostridium difficile* and production of toxins A and B in complex and defined media

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Summary. The ability of several strains of *Clostridium difficile* to grow and to produce toxins A and B in complex and defined culture media has been studied with special reference to the amino-acid composition of the medium. The production of these toxins varied with the strain used and with the composition of the growth medium. Toxin A production was not inextricably linked to production of toxin B since conditions were found in which only one or other toxin was produced.

Introduction

Clostridium difficile is the primary cause of pseudomembranous colitis (Bartlett *et al.*, 1978; George *et al.*, 1978) and one cause of antibiotic-associated diarrhoea (Bartlett *et al.*, 1978). Although the organism is often found in the faeces of healthy neonates, and occasionally, of adults (Keighley, 1981; Viscidi *et al.*, 1981), *C. difficile*-induced diarrhoea is rare in colonised neonates whereas clinical experience suggests that a significant proportion of colonised adults develop diarrhoea (Lishman *et al.*, 1981). Amongst other factors, the nutritional status of the gut may play an important role in the ability of this pathogen to grow and produce toxins at any particular time.

In this paper we describe experiments in which various strains of *C. difficile* were tested for their ability to grow and to produce toxins A and B in a complex medium and in a range of chemically-defined media. Particular attention was paid to the effect of withholding specified amino acids. Furthermore, we wished to establish whether there were conditions in which only one of toxins A and B would be produced, because detection of toxin B (and not toxin A) is currently used in diagnosis of *C. difficile* infections. Moreover, it has been claimed (Lyerly *et al.*, 1983) that toxins A and B are co-produced in a fixed ratio.

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Materials and methods

Strains of *C. difficile*

The following strains were used: 8051, 8089, 8410, 8864 and 23289/78 were clinical isolates from The General Hospital, Birmingham; strains B–1 and BT were clinical and environmental isolates, respectively, provided by Dr S. P. Borriello of The Clinical Research Centre, Harrow, Middlesex. All strains were stored at room temperature in cooked-meat broth.

Culture media

Complex medium consisted of Bactotryptose (Difco) 20% w/v, glucose 0·1% w/v, Na_2HPO_4 0·2% w/v, KNO_3 0·1% w/v in water (tryptic nitrate broth, TNB). This medium was similar to that described by Redmond *et al.* (1985) but with two changes: clindamycin was omitted because it appeared to inhibit toxin production in the defined media described below, and the use of the reducing agent sodium formaldehyde sulphoxylate was unnecessary because all cultures were incubated in an anaerobic chamber with an atmosphere of CO_2 10%, H_2 10%, N_2 80%. TNB was sterilised by autoclaving (15 psi, 121°C, 15 min).

Complete defined medium (table I) was based on that described by Rolfe and Finegold (1979) with two modifications. Haemin was omitted because it had no effect on growth or toxin production and the medium was sterilised by membrane filtration (pore size 0·22 μm). Defined amino-acid-deficient media were prepared by omitting the appropriate amino acid from the complete medium.

All media were pre-incubated in the anaerobic chamber for at least 2 days before inoculation.

Growth conditions

Complete defined medium (in 5-ml volumes) was seeded with several colonies of individual *C. difficile* strains from 2-day-old blood-agar cultures. These broths

Table I. Composition of complete defined medium

Amino acids (g)		Vitamins (μg)		Minerals (mg)	
Histidine	0·1	Thiamine	1000	KH₂PO₄	900
Tryptophan	0·1	Ca-D-Pantothenate	1000	NaCl	900
Glycine	0·1	Nicotinamide	1000	CaCl₂·2H₂O	26
Tyrosine	0·1	Riboflavin	1000	MgCl₂·6H₂O	20
Arginine	0·2	Pyridoxine	1000	MnCl₂·4H₂O	10
Phenylalanine	0·2	p-Aminobenzoic acid	50	(NH₄)₂SO₄	40
Methionine	0·2	Biotin	12·5	FeSO₄·7H₂O	4
Threonine	0·2	Folic acid	12·5	CoCl₂·6H₂O	1
Alanine	0·2	B₁₂	5·0		
Lysine	0·3				
Serine	0·3				
Valine	0·3				
Isoleucine	0·3				
Proline	0·3				
Aspartic acid	0·3				
Leucine	0·4				
Cysteine	0·5				
Glutamic acid	0·9				

To the above constituents were added glucose 5·0 g and sodium carbonate 4·0 g. Water was added to 1L and the medium sterilised by membrane filtration.

were incubated at 37°C in anaerobic conditions for 24 h. They were diluted 1 in 100 in prereduced saline and 20 μl of this diluted suspension was used to inoculate 5-ml volumes of the various test media (TNB, complete defined, and amino acid-deficient) to give final suspensions of c. 10^3 cfu/ml. (Logistical considerations in setting up this type of experiment inside an anaerobic chamber required maximum simplicity of design and ease of operation. The final dilution of spent medium from starter cultures in amino-acid deficient media was 1 in 2500. Thus the carry-over of amino acids was minimal, though not necessarily zero.) The broths were incubated at 37°C in

the anaerobic chamber for 4 days before the culture supernates were collected by centrifugation. Toxin assays were performed on unconcentrated culture supernates.

Toxin assays

Toxin A was measured quantitatively by the direct sandwich ELISA as described by Redmond et al. (1985). This assay can detect 0·005 μg of toxin A/ml. Toxin A concentrations of <0·005 μg/ml were recorded as − in tables II and III.

Toxin B titres were determined by a conventional

Table II. Toxin production by C. difficile in TNB and complete defined media

C. difficile strain no.	Toxin production in				
	TNB†		Complete defined medium		
	Toxin A (μg/ml)	Toxin B (titre)	Toxin A (μg/ml)	Toxin B (titre)	Toxin production pattern*
8410	0·14	160	−	−	I
8864	−	6400	−	20	II
8051	0·20	160	0·11	80	III
23289/78	0·40	80	0·70	80	III
B–1	3·20	2560	0·36	64	IV
8089	11·00	25600	0·28	80	IV
BT	3·00	2000	9·60	4000	V

* See *Results* section.
† The data recorded for toxin production in TNB are typical of four experiments with each strain. Exceptions are strains 8410 and 8051 which were tested once.

Table III. Effect of amino-acid deprivation on toxin production

Medium	BT			B–I			23289/78			Amino acid group*
	Growth	Toxin A (μg/ml)	Toxin B (titre)	Growth	Toxin A (μg/ml)	Toxin B (titre)	Growth	Toxin A (μg/ml)	Toxin B (titre)	
TNB†	+	3·0	2000	+	3·2	2560	+	0·40	80	
Complete defined†	+	9·6	4000	+	0·36	64	+	0·70	80	
Amino acid-deficient‡										
No Pro	−	−	−	−	−	−	−	−	−	
Val	−	−	−	−	−	−	−	−	−	A
Ile	−	−	−	−	−	−	−	−	−	
Leu	−	−	−	−	−	−	−	−	−	
Trp	−	−	−	−	−	−	−	−	−	
Met	−	−	−	+	0·09	16	+	0·06	10	B
Cys	−	−	−	+	0·13	8	+	0·19	40	
Asp	+ +	5·2	1000	+ +	0·40	64	+ +	0·84	80	
Thr	+ +	4·5	2000	+ +	0·31	32	+ +	0·78	40	
Ser	+ +	6·9	2000	+ +	0·19	64	+ +	0·48	40	C
Phe	+ +	12·0	4000	+ +	0·33	64	+ +	0·12	40	
Arg	+ +	3·8	2000	+ +	0·13	16	+ +	0·90	40	
His	+ +	7·0	4000	+ +	0·06	32	+ +	−	−	D
Tyr	+ +	10·8	2000	+ +	0·33	64	+ +	−	10	E
Lys	+ +	5·0	2000	+ +	0·39	128	+ +	−	20	
Glu	+ +	3·2	1000	+ +	0·14	−	+	0·50	40	
Ala	+ +	6·4	4000	+ +	0·16	−	+ +	0·80	80	F
Gly	+ +	8·7	2000	+ +	0·09	−	+ +	0·80	40	

* See *Results* section
† Data from table II.
‡ Amino-acid deficient media were complete defined medium without the named amino acid.

cytotoxicity test with McCoy cells (Redmond *et al.*, 1985). Titrations were performed on doubling dilution series from unconcentrated culture filtrates. Titres were recorded as the dilution that gave a 50% cell-rounding end-point. A titre of < 8 was regarded as not significant and recorded as − in tables II and III.

Qualitative estimation of growth

Cultures were inspected visually after 24 and 96 h. In table III vigorous growth at 24 h is recorded as + +, growth at 96 h as +, and no visible sign of growth at 96 h as −.

Results

Medium preparation and toxin production

Rolfe and Finegold (1979) prepared the defined medium by autoclaving all constitutents except cysteine and sodium carbonate which were filter-sterilised and added later. We found that in medium prepared in this way, growth of the organism was unaffected, but production of toxins A and B was extremely variable. On occasions even strain BT, the most prolific toxin producer on filter-sterilised complete defined medium (see table II), did not produce either toxin in autoclaved defined medium.

Toxin production in TNB and complete defined medium

Toxin production by *C. difficile* grown in TNB and filter-sterilised complete defined medium is summarised in table II. From the strains examined, five different patterns (I–V) of toxin production emerged. Strain 8410 (pattern I) produced small amounts of both toxins in TNB but neither toxin in complete defined medium. Strain 8864 (pattern II) produced high concentration of toxin B in TNB and low concentrations in complete defined media. No toxin A was produced in either medium. Strains 8051 and 23289/78 (pattern III) produced low concentrations of both toxins in both media. Strains B–1 and 8089 (pattern IV) produced high concentrations of both toxins in TNB and lower concentrations of both in complete defined medium. Strain

BT (pattern V) produced high concentrations of both toxins in each medium.

Amino acid-deficient media

Three strains, 23289/78, B–1 and BT, i.e., one representative strain from toxin-production patterns from III, IV and V respectively, were chosen for a more detailed study of amino-acid requirements. Strains with patterns I and II were not used because they produced little or no toxins A or B in complete defined medium.

Several experiments were performed with defined amino-acid deficient media prepared by autoclaving and, as with complete defined medium, the results were variable. The results in table III represent an experiment with media prepared from one batch of filtered stock solutions and are internally consistent. Each vertical column showing growth in defined (complete and amino-acid-deficient) media represents cultures which were seeded with the same starter culture. Thus a negative result could not have been due to the non-viability of the inoculum. Each horizontal row represents a medium of constant composition seeded with each of three strains. Thus a negative result could not have been due to accidental variations in the medium because in every case positive results were obtained with some combination. The only exception to this statement was with respect to group A. Omission of any one of these amino acids resulted consistently in no growth and no toxin production. In each case where either toxin A or B was recorded as 'low' or 'negative', the data were checked several times and found to be consistent within the limits of the tests.

Amino acids could be divided into six groups (A–F) according to their effect on growth or toxin production. Group-A amino acids were absolute requirements of all three strains for growth. Without growth there was no toxin production. Omission of group-B amino acids (sulphur-containing methionine and cysteine) inhibited growth of strain BT. Strains B–1 and 23289/78 grew poorly and produced low yields of both toxins. Omission of group-C amino acids had little or no effect on growth of, or ability to produce toxins by, any of the three strains. Group D consisted of histidine only, omission of which did not prevent growth of strain 23289/78 but totally inhibited production of both toxins. Omission of group-E amino acids suppressed production of toxin A but not of toxin B by strain 23289/78. Omission of group-F amino acids suppressed production of toxin B but not of toxin A by strain B–1.

Discussion

The composition of bacteriological media greatly affected the ability of the three strains of *C. difficile* to grow and to produce toxins A and B. Group-A amino acids—proline, valine, leucine, *iso*leucine and tryptophan were essential for growth in defined medium. Thus the competitive effect of other gut organisms on *C. difficile* could be determined by the availability of these amino acids and the relative efficiencies of amino-acid transport in competing organisms. The absolute requirement for tryptophan may also explain the variability of results observed when complete defined medium was prepared by autoclaving. Tryptophan is acid-labile, especially at high temperature, and may be partially destroyed by autoclaving; other medium constituents may also be heat-labile. This may explain the inability of the strain used by Rolfe and Finegold (1979) to produce toxin (toxin A and toxin B had not then been distinguished) in this medium. Alternatively, their strain could have been similar to strain 8410 which did not produce either toxin in defined medium.

Previously, toxins A and B were thought to be produced at constant ratios irrespective of strain or medium (Lyerly *et al.*, 1983). Within the limits of the accuracy of the two test systems, this may be true for strain BT. However, we have shown here that toxin A can be produced in the absence of detectable toxin B by strain B–1 in glutamate, glycine- and alanine-deficient media, and that toxin B can be produced in the absence of detectable toxin A by strain 8864 in TNB and by strain 23289/78 on tyrosine- and lysine-deficient media.

The work described in this paper shows that the production of toxins A and B by *C. difficile* depends both on the strain used and on the medium in which it was grown. In particular, it would appear that for some *C. difficile* strains there are amino acids of which there are limiting (at present unspecified) concentrations below which growth of *C. difficile* occurs but toxin production is inhibited. We do not imply that the results presented here constitute a rigid biotyping system; considerably more work needs to be done to develop this approach.

Attempts to predict the pathogenic potential of any strain of *C. difficile* in terms of its ability to produce toxins *in vitro* would clearly require that the medium reflected closely the nutritional status of the gut at the time of infection. This would be difficult.

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