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Suppression of toxin production in *Clostridium difficile* VPI 10463 by amino acids

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The impact of various growth conditions on the expression of toxins and other proteins by *Clostridium difficile* VPI 10463 was studied. During non-starved conditions, the rate of toxin synthesis paralleled that of total protein during both exponential growth and stationary phase, and in both defined and complex media. Biotin limitation reduced growth rate and bulk protein synthesis, whereas toxin expression continued, leading to a 50- to 200-fold increase in intracellular toxin levels. Concomitantly, several 22 kDa proteins were up-regulated as revealed by two-dimensional PAGE analysis. The toxin yield was 30-fold higher in peptone yeast extract (PY) than in PY containing glucose (PYG). By contrast, glucose limitation reduced toxin yields by 20- to 100-fold in defined media. By elevating the buffering capacity and bicarbonate concentration, toxin yields were increased by 10-fold in PY and PYG. The high toxin production by *C. difficile* during growth in PY was lowered 100-fold by adding a blend of nine amino acids and several 60–100 kDa proteins were concomitantly down-regulated. It was concluded that toxin expression in *C. difficile* VPI 10463 was not affected by growth rate, growth phase, catabolite repression or the stringent response. Instead the co-expression of toxins and a few specific additional proteins appeared to be influenced by metabolic pathways involving CO_2 assimilation, carboxylation reactions and metabolism of certain amino acids.

Keywords: amino acids, bicarbonate, biotin, *Clostridium difficile*, toxin production

INTRODUCTION

Clostridium difficile is an anaerobic, spore-forming, Gram-positive rod-shaped bacterium that has been identified as an important cause of nosocomial antibiotic-associated diarrhoea (AAD). Despite the fact that *C. difficile* was isolated as early as in 1935 (Hall & O'Toole, 1935) the correlation between the use of antibiotics and *C. difficile*-associated diarrhoea/disease (CDAD) and pseudomembranous colitis (PMC) was not recognized until the late 70s (Bartlett *et al.*, 1977; George *et al.*, 1978). The current view is that suppression of the normal bacterial flora of the human large intestine by the administration of antibiotics allows for overgrowth of *C. difficile*, accumulation of its toxins and onset of CDAD.

Virulent strains of *C. difficile* produce two toxins, A (TcdA or ToxA) and B (TcdB or ToxB), which have been identified as the major virulence factors (Lyerly & Wilkins, 1986). Toxins A and B are the largest bacterial toxins known, with predicted molecular masses of 308 and 270 kDa, and demonstrate a high degree of homology both at the DNA and amino acid level (Barosso *et al.*, 1990; Dove *et al.*, 1990; von Eichel-Streiber *et al.*, 1992). The toxins are endocytosed by the intestinal epithelial cells via coated pits, and both toxins glucosylate members of the Rho protein family of GTP-binding proteins by an UDP-glucose-dependent glucosyltransferase activity. This leads to the disruption of the actin cytoskeleton and cytotoxicity (von Eichel-Streiber *et al.*, 1996; Just *et al.*, 1995a, b). The toxin genes, *tcdA* (*toxA*) and *tcdB* (*toxB*), are in close proximity on the chromosome as part of a 19·6 kb DNA insert. This insert, called the 'toxigenic element' or 'pathogenicity locus', is typical of toxin-producing strains. The sequence homology and the shared enzymic function of toxins A and B suggests that the genes have evolved by gene duplication (von Eichel-Streiber *et al.*, 1992).

The toxigenic element contains five ORFs and one of

Abbreviation: 2-D, two-dimensional.

these, *tcdD* (*txeR*), has been suggested to encode a protein acting as a positive transcription factor for the toxin genes (Hundsberger *et al.*, 1997; Moncrief *et al.*, 1997). Several mRNA species are produced from the toxigenic element, including a polycistronic transcript of approximately 17·5 kb (Hammond & Johnson, 1995; Hammond *et al.*, 1997). The toxin genes are under the control of promoters both in the vicinity and upstream of the coding sequences, explaining the presence of both mono- and polycistronic mRNA (Dupuy & Sonenshein, 1998).

C. difficile has been shown to be highly heterogeneous with respect to toxin production *in vitro* with toxin activity differing more than 100000-fold between strains (Lyerly & Wilkins, 1986), but whether high-toxin-producing strains are more virulent *in vivo* is not known. Environmental conditions affect toxin production in *C. difficile*, and it has been reported that starvation for biotin in a defined medium or for glucose in a rich medium leads to increased toxin yields (Yamakawa *et al.*, 1996; Dupuy & Sonenshein, 1998). To better understand the regulation of toxin expression in *C. difficile*, it is necessary to identify the environmental factors that induce or supress toxin production and the proteins that are co-regulated with the toxins. Here, we monitored in greater detail how toxin production by *C. difficile* strain VPI 10463 was affected by growth rate, growth phase, biotin, glucose, amino acids and different buffering of the medium. Moreover, the proteins that were co-expressed with the toxins were analysed by two-dimensional (2-D) PAGE. The finding that toxin production by *C. difficile* VPI 10463 was markedly lowered by the presence of certain amino acids in the growth medium is discussed.

METHODS

Growth media. Minimal defined medium (MDM), supplemented defined medium (SDM) and complete defined medium (CDM) were modified versions of media described by Yamakawa *et al.* (1994) and Karasawa *et al.* (1995). The defined media were prepared by adding all chemicals (Table 1) to sterile water heated to 60 °C; biotin or glucose were omitted where indicated. The media were kept at 60 °C and equilibrated with an anaerobic gas mixture (10% CO_2, 10% H_2, 80% N_2) for a minimum of 20 min. The media were sterilized using 0·45 μm pore size filters (Acrodisc, Gelman Sciences) and aliquoted into tubes with serum vial-style necks (Bellco Glass) while flushing the tubes continuously with anaerobic gas mixture. The tubes were closed with butyl stoppers and secured with aluminium crimp seals. Pre-reduced, anaerobic and sterilized peptone yeast extract (PY) was purchased from the Karolinska hospital, Stockholm, Sweden. PY supplemented with 0·9% (w/v) glucose is referred to as PYG. Buffered PY and PYG were prepared by adding KH_2PO_4/Na_2HPO_4 or $KH_2PO_4/NaHCO_3/Na_2HPO_4$ at the concentrations given in Table 1. Amino-acid-supplemented PY and PYG were prepared by adding the amino acids present in SDM (C, G, I, L, M, P, T, V and W) or the amino acids A, D, F, H, K, R, S and Y (concentrations as in Table 1).

Strain and inoculum preparation. The toxin-producing *C. difficile* strain VPI 10463 (CCUG 19126; Culture Collection, University of Göteborg, Sweden) was used in all experiments. It was grown anaerobically on blood agar, inoculated into SDM and grown to an optical density of 0·5–1·0 at 420 nm. Glycerol was added to a final concentration of 10% (v/v) and the bacterial culture was aliquoted and stored at −70 °C.

Growth and sampling of *C. difficile*. Aliquots of frozen *C. difficile* were thawed and 0·2 ml was seeded into 20 ml SDM using a 1 ml syringe. To ensure anaerobic conditions during transfer, the syringe was filled with anaerobic gas mixture prior to the transfer. The tubes were placed horizontally on a rotary shaker at 50 r.p.m. and incubated at 37 °C overnight. The next day, the cultures were serially diluted in three 100-fold steps (10^{-6} dilution) into pre-warmed media. When the cultures reached a measurable optical density, 1 ml samples were withdrawn anaerobically at 90 min intervals (45 min for cells growing in PY and PYG). Optical density was measured either at 420 nm (MDM, SDM and CDM) or at 600 nm (PY and PYG) using a Hitachi U-1100 spectrophotometer. In order to compare the OD_{600} with the OD_{420} values, a standard curve was constructed that allowed the transformation of the measurements at 600 nm to the corresponding measurements at 420 nm. The non-linearity between OD_{420} values and cell mass at high cell densities was corrected by using a standard curve obtained by measuring the optical density of undiluted and diluted cultures.

Sample preparation and protein assay. The culture samples were centrifuged at 16000 *g* for 5 min, the supernatants removed and filtered through 0·45 μm pore size filters. Cell pellets were washed once in 1 ml PBS (l^{-1}: 0·2 g KCl, 8 g NaCl, 0·21 g KH_2PO_4, 1·43 g Na_2HPO_4, pH 7·4) centrifuged, stored at −20 °C for a minimum of 60 min, thawed, dissolved in 1 ml sterile water and sonicated on ice for 2 × 30 s at 100 W (Labsonic 1510, B. Braun) with a 15 s pause between the pulses. Cell debris was removed by centrifugation and filtration, and the cell extracts were stored at −20 °C for later analysis. Protein concentration was determined using a kit (Bio-Rad) according to the manufacturer's instructions. BSA was used to obtain a standard curve.

Enzyme immunoassay (EIA) of toxins. Toxins A and B were measured using the Ridascreen *C. difficile* Toxin A/B kit (r-Biopharm) according to the manufacturer's instructions. Thawed samples were diluted in buffer (r-Biopharm) and 50 μl aliquots were added to the wells. A microtitre plate reader (Labsystems Multiscan MCC/340) was used to monitor the absorbance at 450 nm. An A_{450} value of 1·0 was defined as 1 U of toxin.

2-D PAGE. Protein samples were prepared from stationary-phase *C. difficile* cells grown in five different media; PY, PY supplemented with the amino acids present in SDM, PYG, SDM, and in SDM lacking biotin. Cell pellets from 10 ml cultures (except for SDM lacking biotin where 200 ml was used) were stored at −20 °C for a minimum of 60 min, thawed, resuspended in 1 ml sterile water and sonicated on ice for 20 × 30 s at 100 W with a 30 s pause between the pulses. The cell extracts were centrifuged at 16000 *g* for 5 min and 40 μl aliquots of supernatant were mixed with 160 μl buffer containing 9·9 M urea, 4% (v/v) Igepal CA-630, 2·2% (v/v) Pharmalyte carrier ampholytes pH 3–10, 100 mM DTT and 2% (w/v) CHAPS. All protein preparations were stored at −70 °C before use. Protein mixtures were focused at 20 °C on 180 mm IPG Drystrip pH 3–10 or 70 mm IPG Drystrip pH 4–7 (Pharmacia Biotech) using the Multiphor II 2-D gel kit according to the manufacturer's instructions. The second dimension was run by using precast 12–14% gradient gels (Pharmacia Biotech). The gels were stained with silver

Table 1. Composition of defined media

Amino acids	Concn (mg l^{-1})	Minerals, carbohydrates and vitamins	Concn (mg l^{-1})
Tryptophan (W)*	100	$CoCl_2.6H_2O$	1
Methionine (M)*	200	$FeSO_4.7H_2O$	4
Isoleucine (I)*	300	$MnCl_2.4H_2O$	10
Proline (P)*	300	$MgCl_2.6H_20$	20
Valine (V)*	300	$CaCl_2.2H_20$	26
Leucine (L)*	400	$(NH_4)_2.SO_4$	40
Cysteine (C)*	500	KH_2PO_4	900
Glycine (G)†	100	NaCl	900
Threonine (T)†	200	$NaHCO_3$	5000
Histidine (H)‡	100	Na_2HPO_4	5000
Tyrosine (Y)‡	100		
Alanine (A)‡	200	Glucose	2000
Arginine (R)‡	200		
Phenylalanine (F)‡	200	D-Biotin	0·012
Aspartic acid (D)‡	400	Calcium D-pantothenate	1
Lysine (K)‡	400	Pyridoxine	1
Serine (S)‡	400		

* Amino acids in minimal defined medium (MDM).

† Further amino acids added to MDM to give supplemented defined medium (SDM).

‡ Further amino acids added to SDM to give complete defined medium (CDM).

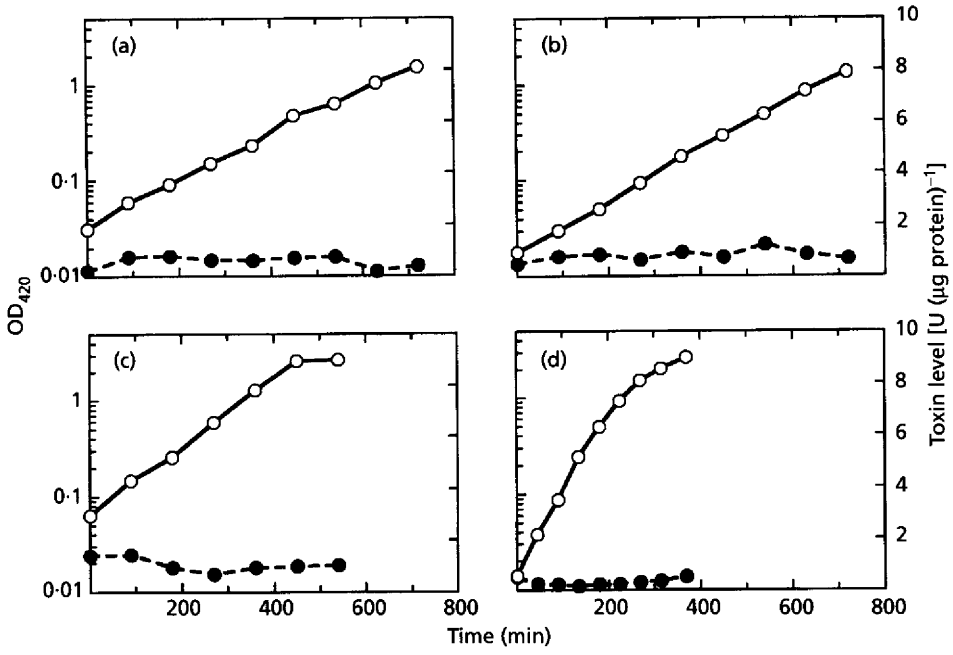

Fig. 1. Growth (○) and intracellular toxin levels (●) of *C. difficile* during exponential growth. Stationary-phase SDM cultures of strain VPI 10463 were serially diluted in three 100-fold steps (10^{-6} dilution) in MDM (a), SDM (b), CDM (c) or PYG (d) and the diluted cultures were incubated at 37 °C. Each panel shows a representative result from three independent experiments.

(PlusOne, Pharmacia Biotech) using a Hoefer automatic gel stainer (Pharmacia Biotech). Chemicals were obtained from Sigma except for Pharmalytes (from Pharmacia Biotech).

RESULTS

C. difficile toxin production displays steady-state kinetics during non-starved growth conditions

High toxin yields by *C. difficile* have previously been obtained during slow growth in a diluted complex medium (Lyerly & Wilkins, 1995), and toxin mRNA has been reported to accumulate during the late exponential phase in a glucose-deficient complex medium (Dupuy & Sonenshein, 1998). Thus, toxin expression may respond to changes in growth rate, growth phase or nutrient concentration. To determine whether the toxin genes respond to growth rate or growth phase, we followed the kinetics of toxin production by *C. difficile* in defined media supporting different growth rates (MDM, SDM, CDM) and one complex medium (PYG). As balanced growth is crucial for basal measurements of physiological parameters in batch cultures (cf. Åkerlund *et al.*, 1995), a small inoculum was used allowing at least eight cell doublings before the onset of the experiment (see legend to Fig. 1). The growth rate was about 0·4, 0·5, 0·7 and 1·6 doublings h^{-1} in MDM, SDM, CDM and PYG, respectively (Fig. 1a–d). The intracellular toxin level, defined as the toxin:protein ratio, was similar in the three different defined media (approx. 1 U μg^{-1}), 2·7-fold lower in PYG ($P < 0{\cdot}01$) as compared to that in SDM, and essentially unchanged during the exponential growth phase (Fig. 1a–d). The total toxin level (intra- plus extracellular toxin:total protein) was only slightly higher in stationary phase (48 h incubation) than during exponential growth (0·5–4 U μg^{-1} depending on the medium, not shown). Thus, neither growth rate nor growth phase had any dramatic effect on toxin production by *C. difficile* in defined or complex media.

Biotin limitation causes growth retardation while toxin production continues

Growth of *C. difficile* in a biotin-limited medium reportedly results in enhanced toxin production (Yamakawa *et al.*, 1996). We further analysed the kinetics of toxin expression in three defined media at different levels of biotin. The biotin concentration in the media was lowered by 10^2- to 10^6-fold of the original, i.e.

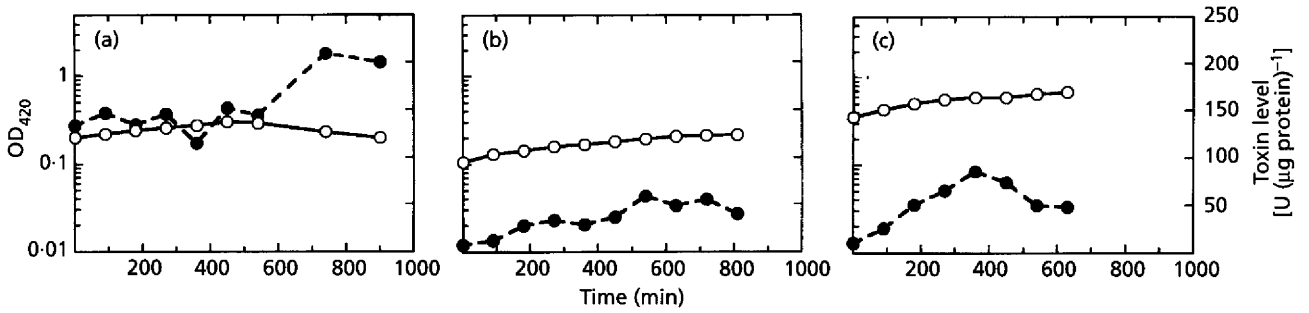

Fig. 2. Growth (○) and intracellular toxin levels (●) of *C. difficile* during biotin limitation. Strain VPI 10463 was inoculated and grown in SDM and serially diluted in three 100-fold steps (10^{-6} dilution) in biotin-depleted MDM (a), SDM (b) or CDM (c) and incubated at 37 °C. The final biotin concentration was 50 fM. Each panel shows a representative result from three independent experiments.

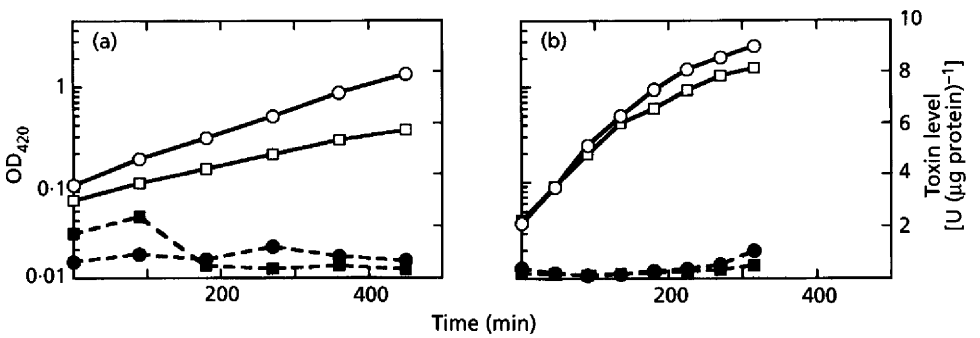

Fig. 3. Growth (○, □) and intracellular toxin levels (●, ■) in *C. difficile* during glucose starvation. Strain VPI 10463 was grown overnight in SDM and diluted 10^6-fold in pre-warmed SDM (a) or PY (b) with (○, ●) or without (□, ■) glucose and incubated at 37 °C. Each panel shows representative results from three independent experiments.

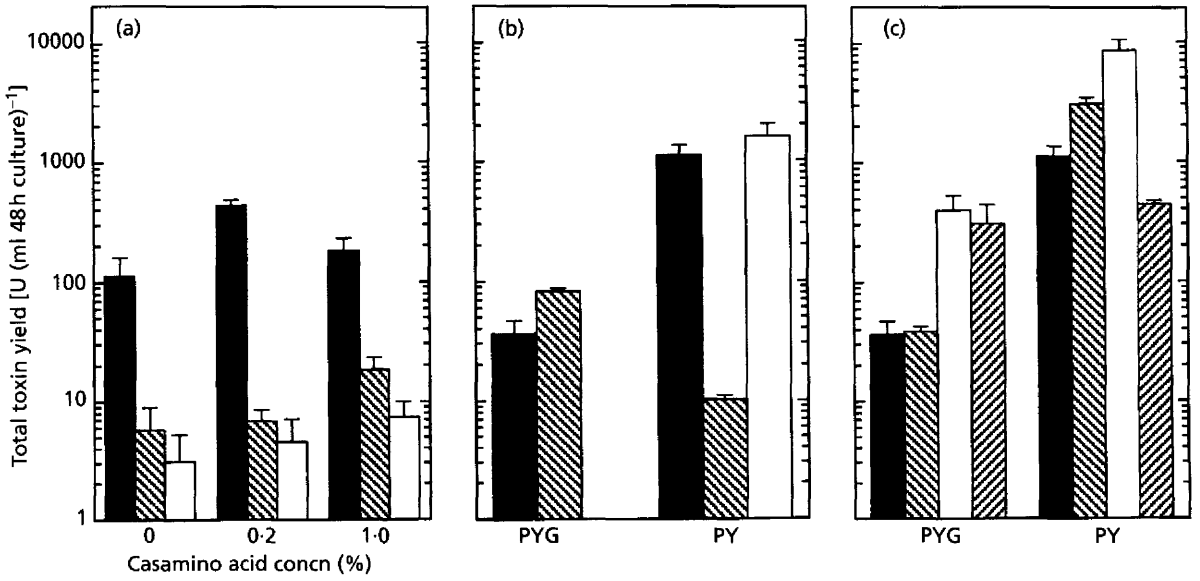

Fig. 4. Effects of glucose starvation, amino acid supplementation and different buffering of the media on total toxin yields in *C. difficile*. Samples (1 ml) of strain VPI 10463 cultures were collected after 48 h incubation at 37 °C and sonicated without prior separation of cells and medium. (a) Toxin yields in SDM with different concentrations of Casamino acids, and with 0·2% (■) or 0·002% (▨) glucose or without glucose (□). (b) Toxin yields in PYG and PY without amino acid supplementation (■) or supplemented with the amino acids C, G, I, L, M, P, T, V and W (▨) or A, D, F, H, K, R, S and Y (□, only determined in PY). (c) Toxin yields in PYG and PY with no buffer added (■), buffered with KH_2PO_4/Na_2HPO_4 (▨) or buffered with $KH_2PO_4/NaHCO_3/Na_2HPO_4$ without (□) or with (▨) the amino acids C, G, I, L, M, P, T, V and W. Concentrations of buffer components and amino acids are given in Table 1. Each bar shows the mean ± SE of three independent experiments. Note that the toxin yields (U ml^{-1}) are not directly comparable with the toxin levels [U (μg protein)$^{-1}$] in Figs 1–3.

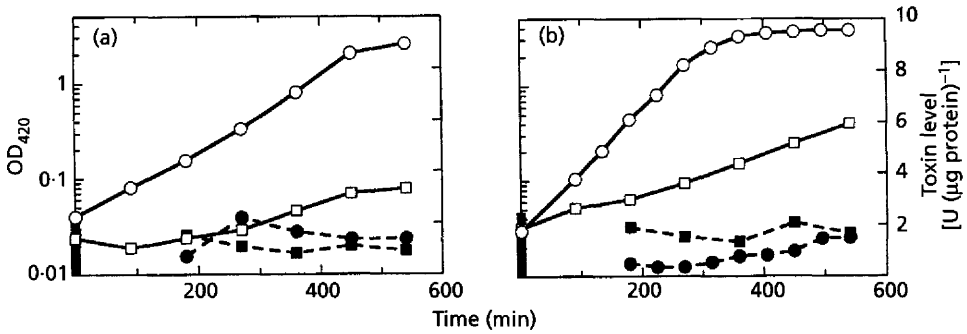

Fig. 5. Effects of nutritional shifts on *C. difficile*. (a) Growth (○, □) and toxin production (●, ■) of strain VPI 10463 during nutritional down-shift. Aliquots (19 ml) of CDM cultures (OD_{420} 0·17) were centrifuged for 3 min at 1250 ***g***. The pellets were resuspended in 0·3 ml CDM, whereafter 0·1 ml was inoculated into 20 ml pre-warmed MDM (□, ■) or CDM (○, ●). The cells were maintained under anaerobic conditions at 37 °C during the entire procedure. (b) Growth (○, □) and toxin production (●, ■) during nutritional up-shift. Cells were grown in SDM to OD_{420} 0·12 and diluted 10-fold in pre-warmed SDM (□, ■) or PYG (○, ●). Intracellular toxin levels (U μg^{-1}) for the starting cultures are represented as histograms at $t=0$. Each panel shows representative results for three independent experiments.

biotin concentrations ranging from 0·5 nM to 50 fM. In the 10^6-fold-diluted cultures, which gave the highest toxin levels, growth was almost arrested at OD 0·2–0·6, i.e. at cell densities 5- to 10-fold lower than in the corresponding biotin-containing cultures (cf. Fig. 2 with Fig. 1). The maximum increase in the intracellular toxin level (200-fold) was obtained in biotin-depleted MDM, whereas the corresponding increase was 50- to 100-fold in biotin-depleted SDM and CDM (Fig. 2a–c). The decline in the toxin level observed in biotin-limited CDM starting at 350 min (Fig. 2c) was caused by release of toxins into the growth medium (not shown). The reason for the release of toxins in CDM but not the other media is not known. Toxin release occurs during

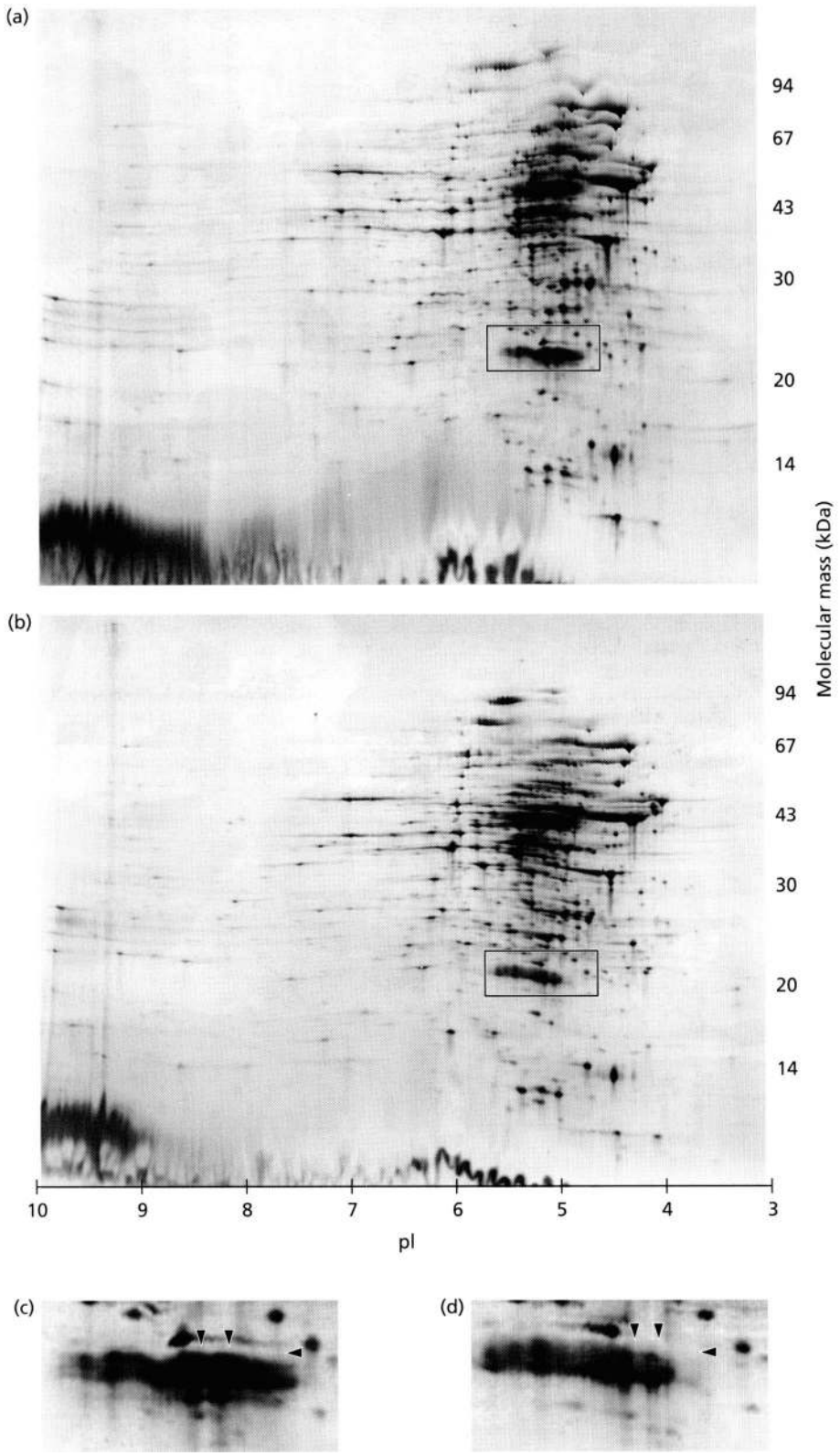

Fig. 6. Protein expression in *C. difficile* during biotin-limited growth. Cultures of strain VPI 10463 were prepared as described in the legend to Fig. 2 and harvested at stationary phase. Ten microgrammes of protein from biotin-limited (a) or non-biotin-limited (b) SDM cultures were visualized by 2-D PAGE. Molecular mass and pI markers are indicated. Enlarged images of boxed areas from (a) and (b) are shown in panels (c) and (d), respectively. Arrows indicate proteins that are up-regulated during biotin-limited growth (i.e. high toxin production). Representative results of three independent experiments are shown.

stationary phase by an unknown mechanism, and it is possible that the higher OD values in biotin-limited CDM (0·5–0·6), as compared to the other biotin-limited defined media (~ 0·2), promotes toxin release. Overall, despite the severe restriction of growth and bulk protein synthesis during biotin limitation, toxin production continued resulting in a dramatic accumulation of toxin within the cells.

Effects of glucose on toxin expression

It was recently shown that transcription of the *C. difficile* toxin A and B genes was induced during the stationary phase in TY medium, but not in TYG, suggesting that the toxin genes are under catabolite repression control (Dupuy & Sonenshein, 1998). To test the generality of this observation, the effect of low glucose on toxin production in a defined medium and in another complex medium was examined. Omitting glucose from SDM or PYG (i.e. PY) resulted in a somewhat slower growth rate but did not affect the intracellular toxin level during exponential or early stationary phase (Fig. 3a–b). As *C. difficile* starts to release the toxins during entry into the stationary phase (cf. Fig. 2c and Lyerly & Wilkins, 1995) we also measured the total toxin yield (intra- plus extracellular toxin units per ml culture) in 48 h cultures. In SDM, low glucose (0–0·002%) resulted in a 35-fold lower total toxin yield as compared to high glucose (0·2–2·0%) (Fig. 4a, 2% glucose not shown). Similar results were obtained in glucose-limited SDM after increasing the amount of fermentable nutrients by adding Casamino acids or 5% PY (Fig. 4a, PY supplementation not shown). In complex medium (PY vs PYG) the opposite effect of glucose depletion was observed (30-fold higher toxin yield, Fig. 4b), confirming the results with TY vs TYG of Dupuy & Sonenshein (1998). Thus, the effect of glucose on *C. difficile* toxin production differed between defined and complex media, implying that glucose limitation does not generally induce toxin production.

Effects of amino acids on toxin expression

C. difficile is, like many other *Clostridium* spp., capable of fermenting amino acids in the absence of fermentable carbohydrates (Mead, 1971). We therefore hypothesized that growth of *C. difficile* in PY might lead to amino acid fermentation and that a resulting latent amino acid starvation could be coupled to toxin induction. Indeed, by supplementing PY with the nine amino acids present in SDM (Table 1) the total toxin yield after 48 h incubation was reduced by 100-fold, as compared to PY cultures (Fig. 4b). In contrast, adding this blend of amino acids to PYG did not alter the toxin yield (Fig. 4b). The dramatic impact of amino acid supplementation on toxin yields in PY cultures was dependent on the amino acids present in SDM as addition of eight other amino acids to PY had no effect on the total toxin yield (Fig. 4b).

An amino acid nutritional down-shift from CDM to SDM (not shown) or MDM did not alter the intracellular toxin levels, although the growth rate markedly slowed down (Fig. 5a). Upon a nutritional up-shift from SDM to PYG, *C. difficile* rapidly adjusted to a higher growth rate, but only minor changes in the intracellular toxin level were observed (Fig. 5b). As was shown in the steady-state experiments (Fig. 1), growth in PYG again resulted in a slightly lower toxin : protein ratio than that in SDM. No change in intracellular toxin level was observed after an up-shift between defined media (from SDM to CDM, not shown). The overall conclusion was that nutritional shifts only had a minor influence on toxin expression.

To summarize, the high total toxin yield from *C. difficile* grown in PY was abrogated by adding a blend of nine amino acids to the medium, indicating that high toxin expression in post-exponential PY cultures was an effect of limited levels of certain amino acids rather than limited levels of glucose.

Increased toxin production at elevated bicarbonate concentration

The pH of 48 h PYG and PY cultures was 5 and 7, respectively, and the low toxin yield observed in PYG could therefore have been caused by acidification of the medium. Increasing the buffering capacity by adding phosphate to PYG resulted in a neutral pH but unchanged toxin yield in 48 h cultures (Fig. 4c). Identical buffering of PY caused a threefold increase in the toxin yield. Thus the lower toxin yield in PYG vs PY cultures was not an effect of low pH. Toxin yields in 48 h PYG and PY cultures increased by 10-fold as phosphate plus bicarbonate was added to the media (Fig. 4c). Supplementing these phosphate-plus-bicarbonate-buffered media with the amino acids present in SDM again reduced the toxin yield in PY but not in PYG (Fig. 4c). Thus, these amino acids again had no impact on the toxin yield when glucose was present.

Expression of *C. difficile* proteins during high and low toxin production

We hypothesized that the high toxin levels during growth in biotin-limited defined media and in PY could be a result of metabolic stress. Therefore, protein expression by *C. difficile* after growth in these media was characterized by 2-D PAGE. About 500 proteins with pIs mostly ranging between 4 and 7 were visualized in bacteria grown in SDM minus biotin (Fig. 6a). Several proteins with molecular masses of approximately 22 kDa and pIs of 5·0–5·5, were found in lowered amounts in the biotin-supplemented SDM culture (Fig. 6b, boxed). The protein pattern in this region was complex and only some of the proteins were down-regulated concomitantly with the toxins (Fig. 6c and d, arrows).

Similar to results obtained in defined medium, about 500 proteins with pIs mostly ranging between 4 and 7 were

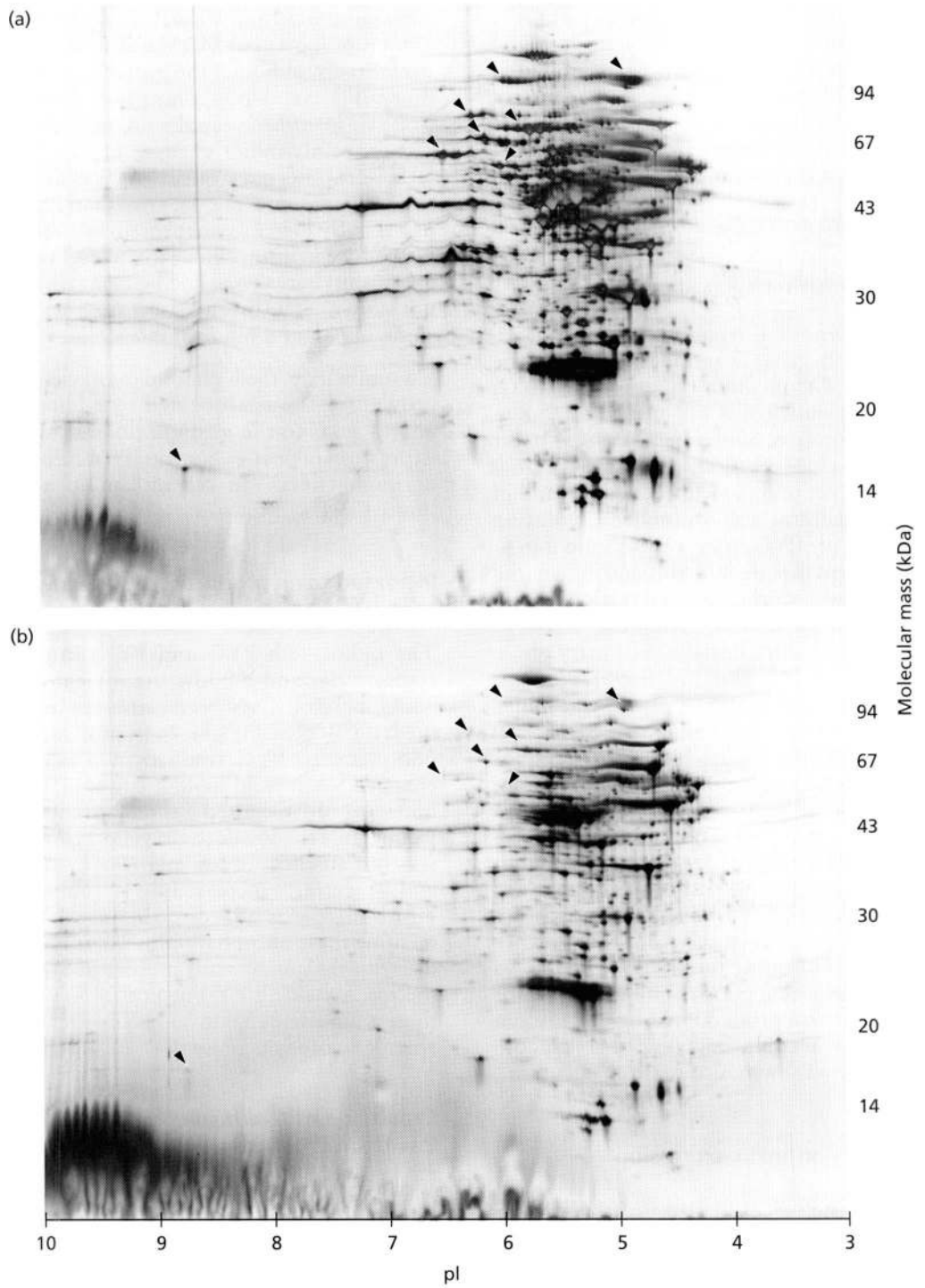

Fig. 7. For legend see facing page.

visualized in bacteria grown in PY (Fig. 7a). Several 60–100 kDa proteins with pIs of 5–7, and one protein of 17 kDa and pI 9 were down-regulated concomitantly with the toxins in PY supplemented with SDM amino acids (Fig. 7b, arrows). The suppression varied from 10- to 200-fold as estimated on digitized images using the Molecular Analyst software (Bio-Rad). However, the quantification of silver-stained proteins is difficult because of the translucent staining that occurs during high protein loads. Thus, the level of highly expressed proteins are often underestimated. The amounts of these proteins were also reduced when toxin production was reduced by the addition of glucose (i.e. in PYG, Fig. 7c, arrows). This difference in protein expression between

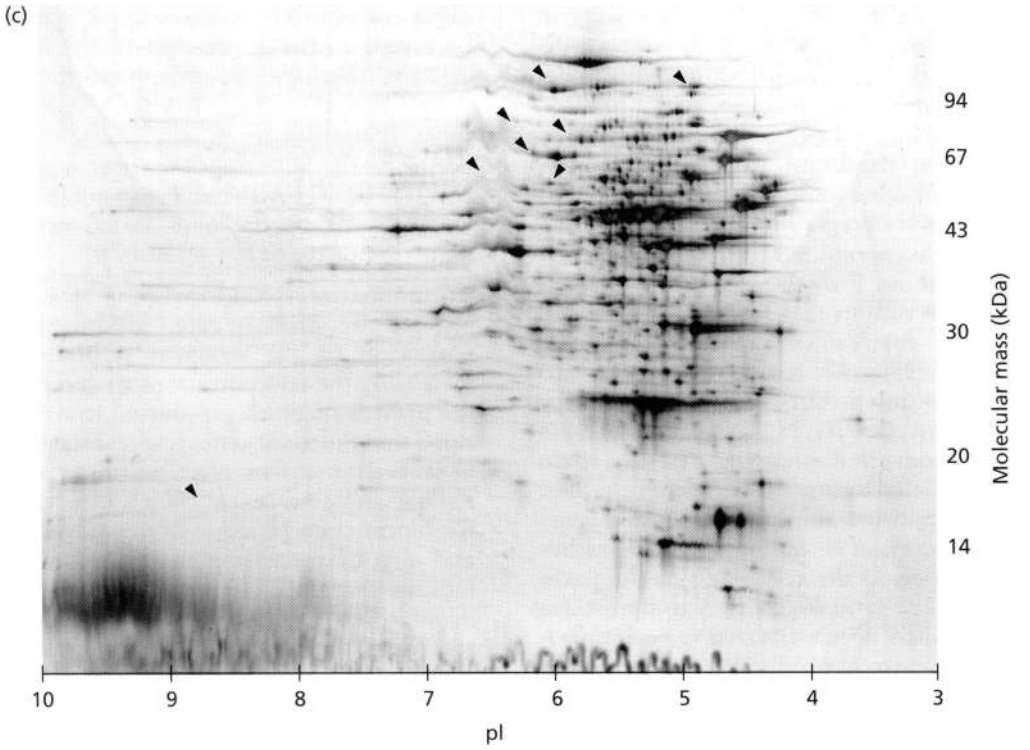

Fig. 7. Protein expression in *C. difficile* grown in PY (a), PY supplemented with the amino acids C, G, I, L, M, P, T, V and W (b) and PYG (c). Ten microgrammes of protein from stationary-phase cultures were visualized by 2-D PAGE. Concentrations of amino acids are given in Table 1. Molecular mass and pI markers are indicated. Arrows indicate proteins that are up-regulated in PY (i.e. high toxin production). Representative results of three independent experiments are shown.

cells grown in PY and supplemented PY was confirmed by 2-D PAGE using a linear 4–7 pI gradient (not shown).

DISCUSSION

We investigated how expression of toxins and other proteins in *C. difficile* VPI 10463 was affected by growth rate, growth phase, biotin, glucose, amino acids and different buffering of the growth medium. The rate of toxin production in *C. difficile* was similar to that of total protein synthesis during the exponential growth phase in various media supporting unrestricted growth, i.e. toxin production was balanced with that of bacterial growth (for a discussion of balanced growth, see Fishov *et al.*, 1995). Little or no change in toxin production was observed during or after entry into stationary phase, indicating that the toxin genes do not belong to regulons normally induced during stationary phase or sporogenesis (Ketley *et al.*, 1984; Ketley *et al.*, 1986).

High toxin production by *C. difficile* has been observed during biotin limitation with an optimum toxin yield at a biotin concentration of 50 pM (Yamakawa *et al.*, 1996). The reduced toxin yields at lower biotin concentrations were probably a result of poor bacterial growth. We found that the intracellular toxin level, i.e. the toxin:protein ratio, a parameter which is independent of the number of bacteria, increased as the biotin level was lowered even further, with a maximum ratio at 50 fM. The cause of the up-regulation of toxin production by *C. difficile* during biotin limitation is not understood. Biotin is required in most CO_2-fixation reactions, for example, the conversion of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase, the first step in fatty acid synthesis (Alberts & Vagelos, 1972; Gucchatit *et al.*, 1974). Fatty acid and protein synthesis appear to be linked in *Escherichia coli* (Magnuson *et al.*, 1993), and may be co-ordinately regulated by the stringent response (Podkovyrov & Larson, 1996). Whatever the mechanism of the repressed bulk protein synthesis in *C. difficile* during biotin limitation, the toxin genes appeared to be uncoupled from this regulation. In addition to the toxins, several 22 kDa proteins were up-regulated during biotin limitation. These proteins may provide clues to the mechanism of toxin regulation and their functions are currently under investigation in our laboratory.

High toxin production by *C. difficile* has been obtained by glucose starvation (TY vs TYG), suggesting that the toxin genes are controlled by catabolite repression (Dupuy & Sonenshein, 1998). We confirmed this observation in PY vs PYG, but found an opposite effect of glucose starvation in defined media (i.e. lowered toxin production), arguing against catabolite repression of toxin production. An alternative explanation for this

observation is that the absence of glucose results in amino acid fermentation and a shortage of amino acids signalling induction of toxin synthesis. Indeed, supplementing PY with the nine amino acids present in SDM (C, G, I, L, M, P, T, V and W) reduced the toxin yield by 100-fold, whereas no reduction was obtained after adding a blend of eight other amino acids (A, D, F, H, K, R, S and Y). We did not observe high toxin yields when the nine amino acids were omitted from defined media. However, as little or no growth was obtained, confirming previous observations that seven of these amino acids are essential for growth in defined media (Karasawa *et al.*, 1995), the result was difficult to interpret. Nutritional down-shifts in defined media by omitting amino acids A, D, F, G, H, K, R, S, T and Y did not elevate toxin production, showing that the toxin genes are not up-regulated during the stringent response. Our results thus supported the hypothesis that starvation for or metabolism of certain amino acids rather than glucose is coupled to the regulation of the toxin genes in *C. difficile*. The ratio of toxins A to B in strain VPI 10463 has previously been estimated to be approximately 3:1 during high toxin production (Hundsberger *et al.*, 1997). We have confirmed this by estimating the amount of toxins A and B by immunoprecipitation during growth in PY and biotin-limited SDM (unpublished). During low toxin production, the amount of toxin A appears to be higher than that of toxin B, although the ratio is difficult to determine because of the low toxin levels. In addition to the toxins, several proteins (60–100 kDa) were up-regulated in PY as compared to PY supplemented with the amino acids or glucose. We are currently identifying the functions of these proteins in order to find the regulon(s) induced during high toxin production.

It was recently reported that the addition of asparagine, glutamic acid, glutamine or lysine reduced toxin yields in *C. difficile* strain KZ 1647 during biotin limitation in a defined medium (Yamakawa *et al.*, 1998). In contrast to Yamakawa *et al.* (1998), we did not observe lower toxin expression in biotin-limited defined media or in PY supplemented with lysine. Moreover, we found no up-regulation of toxin expression when lysine was omitted from a defined medium during nutritional down-shifts. One difference was, however, that higher concentrations of lysine were used in their experiments (1800 mg l^{-1} vs 400 mg l^{-1} in our experiments). Thus, suppression of toxin production by lysine in *C. difficile* may either require higher concentrations of lysine, or alternatively, be strain-dependent.

Elevating the bicarbonate concentration in PY and PYG increased the toxin yields by 10-fold. High bicarbonate concentration and/or CO_2 pressure is likely to affect several biosynthetic reactions, for example biotin-dependent carboxylation. In some anaerobes reductive carboxylation occurs during synthesis of α-ketobutyrate from propionyl-CoA using ferredoxin as a reductant (Buchanan, 1969). In this context, it is interesting to note that α-ketobutyrate is a precursor in isoleucine biosynthesis. Thus, carboxylation and amino acid anabolism is coupled in some bacteria, which in turn might be linked to the expression of the toxin genes in *C. difficile*. Alternatively, as the promoter regions of toxins A and B share similarities with those of UV-induced genes (Hundsberger *et al.*, 1997), the induction may occur via the SOS response. It is, however, difficult to envisage how growth in PY, but not in PY supplemented with glucose or the nine amino acids, would cause induction of the SOS response.

To summarize, toxin expression by *C. difficile* was not affected by growth rate, growth phase, catabolite repression or the stringent response. The fact that increasing the concentrations of certain amino acids in the growth medium suppressed toxin production indicated that the toxin genes respond to regulon(s) involved in starvation for or metabolism of amino acids. The findings that toxin expression was elevated by starvation for biotin, a co-factor used in carboxylation reactions, and also by increasing the bicarbonate concentration indicate that the assimilation of CO_2 or HCO_3^- directly or indirectly regulates the toxin genes. Further studies regarding our finding that toxin production by *C. difficile* can be regulated by administration of amino acids may provide new and important understanding of the regulation of the two toxin genes.

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