Using Phenotype MicroArrays to Determine Culture Conditions That Induce or Repress Toxin Production by *Clostridium difficile* and Other Microorganisms

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Abstract

Toxin production is a central issue in the pathogenesis of *Clostridium difficile* and many other pathogenic microorganisms. Toxin synthesis is influenced by a variety of known and unknown factors of genetics, physiology, and environment. To facilitate the study of toxin production by *C. difficile*, we have developed a new, reliable, quantitative, and robust cell-based cytotoxicity assay. Then we combined this new assay with Phenotype MicroArrays (PM) technology which provides high throughput testing of culture conditions. This allowed us to quantitatively measure toxin production by *C. difficile* type strain ATCC 9689 under 768 culture conditions. The culture conditions include different carbon, nitrogen, phosphorus, and sulfur sources. Among these, 89 conditions produced strong toxin induction and 31 produced strong toxin repression. Strong toxin inducers included adenine, guanosine, arginine dipeptides, γ-D-Glu-Gly, methylamine, and others. Some leucine dipeptides and the triple-leucine tripeptide were among the strongest toxin repressors. While some results are consistent with previous observations, others are new observations that provide insights into toxin regulation and pathogenesis of *C. difficile*. Additionally, we have demonstrated that this combined assay technology can be applied broadly to a wide range of toxin producing microorganisms. This study is the first demonstration of simultaneous assessment of a large number of culture conditions influencing bacterial toxin production. The new functional cytotoxin quantitation method developed provides a valuable tool for studying toxigenic microorganisms and may also find applications in clinical and epidemiological research.

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Introduction

Phenotype MicroArrays (PM) technology provides a simple tool for testing microbial cells [1,2,3] as well as mammalian cells [4] under hundreds or thousands of culture conditions. In 2009, Gardiner and colleagues [5] reported on the use of PM to examine the effect of many culture condition variables on toxin production by the pathogenic fungus, *Fusarium graminearum*. This fungus is a major pathogen on wheat, with severe agricultural and commercial impact. Over many decades of study, no one was able to find *in vitro* culture conditions that would turn on synthesis of the *F. graminearum* trichothecene mycotoxin. However, the use of PM technology provided the breakthrough, indicating that strong toxin induction could be obtained *in vitro* by simply culturing the fungus with arginine, putrescine, agmatine, or guanine as the nitrogen source. Secondarily they found and confirmed that pH 4.5 produced additional induction [6].

A different novel approach was taken by Singh [7] from the Natural Products Discovery Group at Wyeth Pharmaceuticals, who studied substrate utilization effects on secondary metabolite production in fungal strains with promising commercial potential. He used the 95 substrates of the FF MicroPlate combined with scaled-down LC-MS to quantitatively profile the secondary metabolites directly from the microwell culture supernatants. Singh showed this to be a promising approach for both characterization and optimization of secondary metabolite production by fungi.

To expand upon and generalize these works, we have undertaken a study of bacterial toxin induction and repression using an important human pathogenic bacterium and incorporating a new and generally applicable toxin detection method.

In 1978, *Clostridium difficile*, a Gram-positive, spore-forming anaerobic bacillus, was identified as a gastrointestinal pathogen that frequently causes diarrhea and more seriously pseudem-
branous colitis in patients undergoing antibiotic treatment [9,10]. Besides diarrhea, the symptoms of *C. difficile* infection (CDI) include abdominal pain, fever, loss of appetite, nausea, toxic megacolon, and even perforations of the colon and sepsis. Death occurred occasionally. With the emergence of hypervirulent strains, the mortality rate of CDI has risen dramatically. Among serious cases, 15,000–20,000 patients die annually from CDI in the United States [11]. This bacterium is also an important animal pathogen [12].

*C. difficile* is a genetically diverse species with a highly dynamic genome that seems to be evolving rapidly [13,14,15,16,17]. This genetic diversity may be the result of horizontal gene transfer, point mutations, inversions, and large-scale recombination of core chromosomal regions over considerable phylogenetic distance [13,14,15,16]. Disease-causing isolates have arisen not from a single lineage but multiple lineages, suggesting that virulence evolved independently in multiple highly epidemic lineages [13]. These recent findings have provided invaluable insights and significantly advanced our understanding of *C. difficile* pathogenesis and epidemiology.

During the past decade, the prevalence and severity of CDI has increased dramatically worldwide [11,18,19,20,21,22]. The emerging epidemic of “hypervirulent” isolates represented by ribotype 027 (also called BI/NAP1/027), which are variant strains of toxino type III, have been identified as a major culprit in hospital or hospital associated CDI outbreaks [11]. Comparative genomic analyses showed that the epidemic 027 strains have gained 234 additional genes during the past two decades, which may account for their epidemic proficiency and their higher case-fatality ratio [15,16].

Nevertheless, the central issue in the pathogenesis of *C. difficile* is its major virulence factors, which have long been linked to the two large toxins, A and B. The cause-effect relationship between the toxins and the pathological changes they engender in animal cells, the cytopathic effects (CPE), have been shown to be due to inactivation of Rho-GTPase through glucosylation by the toxins [23,24,25,26]. The essential roles the toxins play in *C. difficile* pathogenesis have also been demonstrated in multiple animal models [27,28,29,30,31,32] and in clinical settings [33,34]. Antibodies against toxins A and B as a supplemental treatment to antibiotic regimens have been shown to reduce recurrence of CDI in patients [35,36] and to protect intoxicated animals [36]. Identification of *C. difficile* toxin A or B in patients’ diarrheal stool is critical and required for diagnosis of CDI [37]. The quality and quantity of the toxins are directly or indirectly determined or regulated by multiple factors such as genetic, environmental, nutritional, and metabolic status. Therefore, monitoring functional toxin production is fundamental in studies of pathogenesis and epidemiology as well as in clinical diagnosis and treatment of CDI.

Cell-based cytotoxicity assay (CCTA) is traditionally regarded as the gold standard assay for *C. difficile* cytotoxin and serves as the reference for other toxin assay methods [38]. This assay looks for toxin induced CPE by microscopic detection of a shift from normal to “rounded” morphology using a toxin-sensitive adherent mammalian cell line (an indicator cell, e.g., CHO, Vero, HT-29, foreskin or others) and then verifies that the CPE is prevented by a specific toxin-neutralizing antibody. This gold standard assay is a true test for functional cytotoxicity regardless of whether the DNA coding sequence of the toxin or the sequences of regulatory proteins are mutated. Given that *C. difficile* has an extremely dynamic genome [13,14,15,16,17], it is critical to have a reference assay that directly tests the toxin’s true biological activity. Evidence has shown that, in addition to other factors, virulence is dependent on the autoactivation of a toxin cysteine protease [39,40,41,42]. It is also dependent on the ability of the infected host cell to S-nitrosylate C. difficile toxins, which attenuates virulence by inhibiting toxin self-cleavage and cell entry [43]. Toxin B from hypervirulent strains (TcdBHv) undergoes acid-induced conformational changes at a pH much higher than that of toxin B from historical strains (TcdBHist), which makes TcdBHv enter the cell more rapidly [44]. Further, TcdBHv is autoprocessed more efficiently than TcdBHist [45], which may explain why TcdBHv causes increased cytotoxicity. Understandably, such functional differences of the toxin may not be detected by PCR-based toxin assays, or by ELISA- or Western Blot-based assays which do not necessarily measure toxin function. However, CCTA can detect differences that other methods cannot because it is a direct functional and phenotypic assay of toxins.

Although authoritative, traditional CCTA also has some pitfalls, including laborious and time consuming steps (usually 2–3 days for direct toxin assay of a faecal sample, not including the culturing step), difficulties in quantitation due to subjective interpretation (grading cell rounding), and requirements for tissue culture facilities and well trained technical staff [46]. As traditional CCTA is not highly standardized, interpretation of the cytotoxic or cell-rounding activity will be variable [46]. For example, some consider 50% cell rounding a positive reaction [47,48] while others consider 100% cell rounding a positive reaction [49], making it difficult to compare the results among various laboratories and studies.

We have used a different approach to overcome the inconvenience and limitations of the traditional CCTA assay cited above. To do so, we have taken advantage of Phenotype MicroArray (PM) technology, which can now be employed with both microbial and mammalian cells. In addition to *C. difficile*, we employ a sensitive mammalian indicator cell line (CHO-k1 or Vero cells) as required by CCTA. Combining microbial and mammalian assay methodologies has allowed us to develop an efficient, reliable, highly informative, and quantitative method to measure *C. difficile* toxin production under hundreds to thousands of different culture conditions. Our new hybrid approach enables high throughput evaluation of the role of environmental factors in stimulation or repression of toxin production by *C. difficile*. Given its high sensitivity and reliability, it may potentially be used for direct clinical faecal sample measurement of the toxins. The same approach can be further generalized to study toxins or other secondary metabolites produced by microbial cells.

Here, we report on toxin production of *C. difficile* type strain ATCC 9689 under hundreds of culture conditions, including variations of basic cellular nutritional components for carbon, nitrogen, phosphorus, and sulfur (768 culture conditions, PM1–8). Also, we demonstrate that this same approach is successful with other toxin-producing clostridia as well as toxin-producing aerobic bacteria.

**Results**

**Development of *C. difficile* toxin assays in 96-well format**

For this study, we purchased purified *C. difficile* toxins A and B which are commercially available in lyophilized form. Once the toxins are dissolved into buffer solutions, their potencies decrease rapidly with noticeable loss by the next day, even if the solutions are stored at 4°C. So, to make standard titration curves for quantification purpose, we used exclusively freshly dissolved purified toxins. We observed that the cytotoxic potency of the supernatants collected from the PM panels also decreased over time. Therefore, the assays of the toxins produced by *C. difficile* strains in PM panels (96-well format) were always carried out with
fresh preparations of the supernatants, usually on the same day of collection.

Purified standard *C. difficile* toxin B (Listlab) was used to optimize and calibrate two assays. In a morphological assay the commercial toxin caused cytopathic effects (CPE), seen microscopically as cell rounding changes, in both CHO-k1 and Vero cells. The observed CPE was active in a concentration-dependent manner. The lower panel of Figure 1A shows an example of a toxin B titration with CHO-k1 cells. Purified toxin A (Listlab) was much less potent than toxin B against both cell lines (data not shown).

The CHO-k1 and Vero cells could also be employed in a colorimetric assay using Biolog redox dye MB and an OmniLog instrument to quantitatively measure the degree of intoxication of the cells by the toxin. In this assay also, toxin B-treated cells showed concentration-dependent intoxication of cells that resulted in decreasing rates of color formation as the killed cells were incapable of dye reduction and the dying or injured cells were compromised in the reduction (lower panel of Figure 1B, Figure 1C, Materials and Methods). Therefore, increasing cell rounding in the morphological assay was correlated with decreasing dye reduction rate in the colorimetric assay (Figures 1A, 1B, 1C). Neutralization of toxin B with anti-toxin B polyclonal antibodies IgY (Gallus Immunotech) provided complete protection of the indicator cells in both assays (Figures 1A, 1B, 1C).

The colorimetric dye reduction provided a quantitative assay of toxin over a ≥5 log concentration range. From serial titrations of standard toxin B, the dye reduction rates by mammalian cells were calculated using PM Analysis Software. Regression analysis on known (prepared) concentrations of toxin B and corresponding dye reduction rates by CHO-k1 cells could be accurately fit to several regression equations over a range of serial titrations of toxin B, from 800 ng/ml (~2963 pM) down to approximately 0.122 ng/ml (~0.45 pM). The predicted toxin concentrations calculated from the regression equations were very close to the prepared concentrations (Table 1).

**Figure 1.** Cytotoxicity and neutralization assay of purified *C. difficile* toxin B with CHO-k1 cell line. Serial 3-fold titrations of standard toxin B (ng/ml) in the presence or absence of 2.5 μg/ml neutralizing antibodies IgY specific for toxin B. (A) CHO-k1 cell morphology changed to rounded shapes by toxin B in a dose dependent manner (the lower panel). This was prevented by the neutralizing antibodies (upper panel). (B) CHO-k1 cell dye reduction was reduced by toxin B also in a dose dependent manner (the lower panel), which corresponds to the cell morphological changes. This effect was also prevented by the neutralizing antibodies (the upper panel). (C) Quantification of the dye reduction changes by toxin B, with (Red) or without (Dark blue) 2.5 μg/ml neutralizing antibodies IgY. Light blue: toxin B with control antibodies IgY-010; Purple: no toxin B with no antibodies; Green: no toxin B with control antibodies IgY-010.

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Toxin production under different PM culture conditions

Production at high levels of = 72 h. experiments indicated that toxin production was low at 24 h and thereafter tended to decrease at 48 h and further decrease at 72 h. supernatants) from various PM culture conditions (Figure 2). was also observed with unpurified toxin preparations (filtered between the CPE and dye reduction rate of the intoxicated cells with indicator cells (Figures 2, 3A, 3C, 3D). As seen in the case of purified standard toxin B (Figure 1), the inverse correlation with the indicator cells (Figures 2A, 2B, 3B). Control experiments (no intoxication) were also performed to show that the 768 chemicals themselves did not induce toxicity to the CHO-k1 cells (data not shown). To examine the effect of culture conditions on toxin production, C. difficile cultures could intoxicate indicator cells and be effectively neutralized by anti-toxin B antibody. Both CHO-k1 and Vero cells were intoxicated by crude toxin preparations of C. difficile strain ATCC 9689 supernatants from different culture conditions of the PM panels (Figure 2B). The morphological changes of the CHO-k1 cells were identical to those caused by purified standard toxin B (Figures 1A, 2A, 2B, 3B). However, the levels of toxin production in the supernatants were distinctly different under different PM culture conditions. This is clearly shown both by the cell culture toxicity assay (Figures 2A, 2B, 3B) and by the dye reduction assay with the indicator cells (Figures 2, 3A, 3C, 3D). As seen in the case of purified standard toxin B (Figure 1), the inverse correlation between the CPE and dye reduction rate of the intoxicated cells was also observed with unpurified toxin preparations (filtered supernatants) from various PM culture conditions (Figure 2). These adverse effects of unpurified toxin preparations on cell morphology and dye reduction were also specifically prevented by anti-toxin B IgY polyclonal antibodies (Figures 3A, 3B, 3C, 3D).

The cell mass under most PM culture conditions peaked at 24 h and thereafter tended to decrease at 48 h and further decrease at 72 h (Materials and Methods, Figure S1). In contrast, initial experiments indicated that toxin production was low at 24 h and tended to peak around 72 h.

### Table 1. Predicted concentrations of C. difficile toxin compared to the true concentrations in cell culture assay medium.

| Prepared toxin Conc. (ng/ml)* | Dye Reduction Rate (x)² | Regression Equation | R² | Predicted Toxin Conc. (ng/ml) y= |
|-------------------------------|-------------------------|-------------------|---|-------------------------------|
| 800.000                       | 28.715                  | y=10.488x²–78.989x+14692 | 0.998 | 799.193 |
| 266.667                       | 32.365                  | ibid.              | ibid. | 272.231 |
| 88.889                        | 34.955                  | ibid.              | ibid. | 67.808 |
| 29.630                        | 35.400                  | y = 7E+08x^{-4.7608} | 0.980 | 29.553 |
| 9.877                         | 44.945                  | ibid.              | ibid. | 9.484 |
| 3.292                         | 60.030                  | ibid.              | ibid. | 2.391 |
| 1.097                         | 68.310                  | y = 5E+62x^{-34.1577} | 0.999 | 1.093 |
| 0.366                         | 70.650                  | ibid.              | ibid. | 0.346 |
| 0.122                         | 72.845                  | ibid.              | ibid. | 0.122 |

*Standard purified C. difficile B toxin.

The cell mass under most PM culture conditions peaked at 24 h and thereafter tended to decrease at 48 h and further decrease at 72 h (Materials and Methods, Figure S1). In contrast, initial experiments indicated that toxin production was low at 24 h and tended to peak around 72 h.

### Toxin production under different PM culture conditions

To examine the effect of culture conditions on toxin production, C. difficile ATCC 9689 was cultured in PM1–8 which constitutes 768 culture conditions with variations in the basic metabolic nutrients, C, N, P, and S (Table S1) for 72 h before toxin-containing supernatants were collected. CHO-k1 cells treated with the 768 C. difficile supernatants showed different degrees of CPE (Figures 2A, 2B, 3B). Control experiments (no C. difficile control) were also performed to show that the 768 chemicals themselves did not induce toxicity to the CHO-k1 cells (data not shown). On statistical analysis (t-test) we consider the differences of dye reduction rate between the two groups (C. difficile supernatant group and no C. difficile control group) to be significant only if P<0.05. There were 544 out of 768 culture conditions that significantly induced C. difficile toxin production (Table 2). Of these 544 culture conditions, 89 (16.4% of 544) induced toxin production at high levels of = 420 ng/ml (Tables 2, 3, S2), 192 (35.3% of 544) gave a middle range level of = 42 but <420 ng/ml (Tables 2, 4, S2), and 262 (48.2% of 544) gave a low level range of toxin production, >2562 but <42 ng/ml in the supernatants (Table 2). There were 224 out of 768 culture conditions that were not statistically significant in inducing toxin production (P>0.05 except one PM7 A4-Leu-Trp, P = 0.048). Of these 224, 30 (13.8% of 224) produced lowest toxin levels (<0.122 ng/ml in CHO-k1 cell assay medium, or possibly 0 ng/ml) (Tables 2, 5, S2).

With glucose as the carbon source, a variety of nitrogen sources from PM3, 6, 7, and 8 gave highest toxin productions (>420 ng/ml) (Figures 4A, 4C, Tables 3, S2). The nitrogen sources were the predominant class of high toxin level inducers compared to other categories (e.g., carbon, phosphorus, sulfur sources, etc.) of PM culture conditions. The most powerful C. difficile toxin inducers (>2,100 ng/ml) were found in nucleobases, nucleosides, dipeptides, and amine compounds, which included adenosine, guanosine, arginine dipeptides, γ-D-Glu-Gly, methylamine and others (Figure 4A, Tables 3, S2). Among amino acids as nitrogen sources, L-proline, L-histidine, D-aspartic acid, L-citrulline, L-lysine, and D-lysine were also seen in the group of high toxin inducers (Figure 4C, Tables 3, S2). Overall, the highest toxin inducer was adenine as a nitrogen source in PM3, which exceeded the upper tested concentration of the standard purified toxin B in this study (800 ng/ml). This corresponds to 16,800 ng/ml or greater (with a 21-fold dilution factor applied) in the bacterial supernatant (Figure 4A, Table 3). Guanosine as a nitrogen source induced the second highest toxin production of C. difficile ATCC 9689 (Figure 4A, Table 3). These were followed by xanthosine, thymine, and thymidine, which induced toxin levels >7,000 ng/ml, and by others including cytosine, cytidine, uracil, uridine, and xanthine. The purine metabolic uric acid also stimulated high toxin production (4288 ng/ml) (Figure 4A, Table 3). Interestingly, whereas multiple arginine dipeptides were among the very top toxin inducers (Figure 4A, Table 3), arginine by itself was not, although it was among the category of high toxin producers (Figure 4C, Table 3). It induced ~14–22 times lower than the top toxin-inducing arginine dipeptides (Figures 4A, 4C, Table 3). Presumably it is not taken up as well as the arginine-containing peptides or arginine dipeptides as intact molecules work differently than arginine itself in stimulating the toxin production.
Carbon sources from PM1 and 2 gave lower levels of toxin production and were less frequent toxin inducers. Though weaker inducers than the nitrogen sources in PM3,6,7,8, D-threonine, N-acetyl-D-glucosamine, L-alanine, Ala-Gly, fumaric acid, L-serine, and Gly-Asp were the highest toxin inducers of the carbon sources in panels PM1–2 (Figure 4B), and induced toxin levels of greater than 1500 ng/ml. Phosphorus and sulfur sources from PM4 were not seen as significant toxin inducers with C. difficile ATCC 9689, although cytidine 3',5'-cyclic monophosphate as a P-source in PM4 induced a middle range (320 ng/ml) of toxin production.
None of the culture conditions in PM5, with low levels of potentially stimulatory nutrients, showed significant toxin induction or repression.

The other interesting class is the PM substrates that gave lowest levels of \textit{C. difficile} toxin production or no toxin at all (<0.122 ng/ml in CHO-k1 assay medium, or correspondingly <2.562 ng/ml

\textbf{Figure 3. Cytotoxicity and neutralization assays of toxin prepared from \textit{C. difficile} ATCC 9689 grown in PMs.} Anti-toxin B polyclonal antibodies IgY were used throughout at 2.5 μg/ml for neutralization assays. A. Representative cell-based cytotoxicity and neutralization assays with redox dye MB in PMs 1 and 3 with CHO-k1 cells. B. Typical CHO-k1 cell morphological changes with or without anti-toxin B IgY. PM3: A8 = L-Arginine, B9 = L-Proline, G1 = Xanthine, F2 = Adenine. C. Dye reduction signals by CHO-k1 cells automatically and kinetically collected by OmniLog instrument. The numbers shown are means of dye reduction rates of replicas of each well in PM3, calculated by PM Analysis Software. D. Histograms of the same data of the dye reduction rates as in C. Tan: no \textit{C. difficile}; Red: \textit{C. difficile}; Green: \textit{C. difficile}+anti-toxin B IgY.

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in bacterial supernatant), which were indistinguishable from no C. difficile control (Table 5). The concentrations were so low that they were out of the reliable range of dye reduction measurement. Notable among this group were several leucine dipeptides and the leu-leu-leu tripeptide (Figure 5, Table 5). Given the average toxin production of 57 ng/ml in the bacterial supernatant (or 2.690 ng/ml in the cell assay medium) from the no PM substrate control and 51 ng/ml (or 2.423 ng/ml in the cell assay medium) from all PM substrate controls of PM 6 through 8 (Figure 5, Table 5), and also given that the bacterial mass under those conditions were all comparable (Table 5), the substrates that gave very low or no toxin production may in fact repress toxin production by C. difficile.

Experiments were also performed to compare the results of the type strain of C. difficile to the toxinotype strain VPI 10463 (ATCC 43255). These experiments showed that the toxin levels were greatly elevated in the toxinotype strain which we estimate at 100–1000 times higher than ATCC 9689 (data not shown).

Additional experiments were performed to demonstrate the general applicability of this assay technology to diverse toxin producing bacteria. Using CHO-k1 or Vero cells we could demonstrate variable toxicity from the PM panel supernatants of C. perfringens, C. tetani, C. sordellii, Bacillus cereus, Escherichia coli O157 (Vero), Shigella dysenteriae (Vero), and Listeria monocytogenes (Figure 6).

Discussion

Reliable, quantitative, and robust assays for functional toxins are essential in scientific research and clinical practice. The roots of our new assay extend back by several decades. In 1983, Mosmann published a highly cited, quantitative and colorimetric assay using the tetrazolium salt MTT to measure mammalian cell proliferation and cytotoxicity [50]. Since then, it has been applied in numerous research studies. In addition to its applications in anti-cancer drug research and other toxicity studies, it has also been applied to bacterial cytotoxin studies [51] including measurement of cytotoxicity of C. difficile toxin [43,52,53,54]. Rothman similarly reported quantitation of cell responses to C. difficile toxin by crystal violet staining of mammalian cells [55]. The application of colorimetric dyes significantly reduces the labor time because quantitation of color change avoids the laborious and subjective counting of rounded cells. However, the prior colorimetric assays are also inconvenient in that they require solubilization of dye and reading of the plates manually with a microplate reader. This compromises the efficiency of the assays and does not allow one to collect high throughput kinetic data for analysis.

To develop a new, reliable, quantitative, and robust cytotoxicity assay, we started with the gold standard approach by observing the reliable morphological changes of intoxicated cells by C. difficile toxin B, the cell rounding, or cytopathic effect (CPE). We confirmed that the cell rounding caused by C. difficile toxin B is concentration-dependent and specifically prevented by IgY anti-toxin B polyclonal antibodies. Then, we employed a new proprietary Dye Mix MB [4], that forms a water soluble formazan and does not require solubilization. We also employed the OmniLog instrument which enables the automatic collection of kinetic dye reduction data directly from all wells of 96-well plates in a high throughput format and calculated reduction rates using PM Analysis Software. This enabled measurement of the CPE as decreased cell viability resulting in decreased redox dye reduction rates. Through quantitative analysis, we showed that the CPE is inversely correlated with dye reduction rate of the cells: the stronger the CPE, the lower the dye reduction rate. Furthermore, both toxin-induced CPE and decreased dye reduction rate were specifically and simultaneously prevented by the anti-toxin antibodies. The close correlation between the predicted toxin concentrations calculated from the regression equations and the prepared concentrations (Table 1) indicates that the equations obtained using the standard toxin are reliable and accurate over a wide range (>3 logs) of toxin concentration. Thus, the basis of determining the levels of C. difficile toxin production under various culture conditions in this study has been established.

It is estimated that toxin B of C. difficile is 1000 times more potent than toxin A [56]. We also observed that the indicator cell lines (CHO-k1 and Vero) were much more sensitive to toxin B than to toxin A (data not shown). Because of this large difference, trace or equivalent amounts of toxin A contamination in toxin B preparations would be of no consequence in the cell rounding assay [56]. Therefore, in this study, we employed exclusively IgY anti-toxin B polyclonal antibodies which were capable of completely protecting the cells from purified standard toxin B (Figure 1). As expected, the anti-toxin B IgY almost completely protected the cells from the crude toxin preparations collected from 96-well PM panels as well (Figures 3A, 3B, 3C, 3D).

As a cell-based cytotoxicity assay, this new method has gold standard reliability and makes the traditional cytotoxicity assay objectively quantifiable, more efficient, shorter in turn-around time (1 day rather than 2 or 3 days), and amenable to high throughput testing. By eliminating subjective scoring of cell rounding, it allows results from different laboratories to be compared.

Combining this toxin assay method with various PM culture conditions (96-well plates) provides another unique advantage over the traditional methods used in toxin research. It allows scientists to simultaneously study hundreds to thousands of culture conditions.

Table 2. C. difficile toxin production ranges and statistics under different culture conditions from PM1–8.

| Toxin concentration range (ng/ml) | Number of PM conditions (P<0.05) | Number of PM conditions (P>0.05) | Total |
|----------------------------------|----------------------------------|----------------------------------|-------|
| > = 420                          | 89                               | 0                                | 89    |
| > = 42, < 420                    | 192                              | 10                               | 202   |
| >2.562, <42                      | 262                              | 184                              | 446   |
| <=0.122                         | 1                                | 30                               | 31    |
| Total                            | 544                              | 224                              | 768   |

*For measurable toxin levels, the numbers indicate toxin concentrations in supernatants of C. difficile grown under different PM conditions.

The P values were obtained from t-test on the dye reduction rates by CHO-k1 cells in the presence vs absence of C. difficile supernatants collected from different PM conditions.

Below the measurable limit in CHO-k1 cell assay medium of 0.122 ng/ml. Note that the concentration of the toxin in the cell assay medium is 21-fold diluted from the bacterial supernatant (see Materials and Methods).

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| Plate panel | Well | Chemical | Category | C. difficile Mass* | Toxin (ng/ml)b | P valuec |
|-------------|------|----------|----------|------------------|----------------|----------|
| PM03        | F02  | Adenine  | nucleobase| 0.031            | >16800         | 2.10E-04 |
| PM03        | F07  | Guanosine | nucleoside | 0.0092           | 10402         | 2.45E-05 |
| PM06        | B07  | Arg-Asp | dipeptide | 0.0444           | 10371         | 2.32E-07 |
| PM08        | G02  | γ-D-Glu-Gly | dipeptide, γ- | 0.0519           | 10122         | 3.34E-03 |
| PM06        | B05  | Arg-Ala | dipeptide | 0.0456           | 10080         | 5.70E-08 |
| PM03        | G02  | Xanthosine | nucleoside | 0.0516           | 9499          | 6.36E-09 |
| PM06        | B08  | Arg-Gln | dipeptide | 0.0476           | 8912          | 4.54E-08 |
| PM06        | C05  | Arg-Tyr | dipeptide | 0.0455           | 8819          | 7.07E-05 |
| PM06        | F10  | His-Pro | dipeptide | 0.0481           | 8568          | 6.08E-07 |
| PM06        | C04  | Arg-Trp | dipeptide | 0.0499           | 8423          | 7.81E-07 |
| PM08        | F04  | β-Ala-His | dipeptide, β- | 0.0473           | 8265          | 3.15E-06 |
| PM06        | B06  | Arg-Arg | dipeptide | 0.0435           | 8229          | 4.65E-07 |
| PM03        | F08  | Thymine  | nucleobase | 0.0338           | 7749          | 2.83E-09 |
| PM06        | C03  | Arg-Ser | dipeptide | 0.0448           | 7608          | 1.85E-08 |
| PM07        | D08  | Pro-Hyp | dipeptide | 0.045            | 7523          | 1.04E-06 |
| PM03        | F09  | Thymidine | nucleoside | 0.0384           | 7336          | 3.22E-08 |
| PM07        | E07  | Ser-Pro | dipeptide | 0.0472           | 7136          | 2.23E-07 |
| PM06        | F08  | His-Lys | dipeptide | 0.0453           | 6993          | 1.02E-07 |
| PM06        | C02  | Arg-Phe | dipeptide | 0.0467           | 6541          | 2.70E-07 |
| PM03        | D05  | Methylamine | amine | 0.0398           | 6537          | 1.61E-04 |
| PM07        | F07  | Trp-Arg | dipeptide | 0.0628           | 6468          | 9.53E-07 |
| PM03        | G08  | γ-Aminobutyric acid | amino fatty acid, γ- GABA | 0.0375 | 6529 | 4.45E-11 |
| PM03        | D07  | N-Butylamine | amine, N- | 0.0407 | 5679 | 3.74E-04 |
| PM07        | A07  | Lys-Arg | dipeptide | 0.0502           | 5536          | 5.20E-06 |
| PM03        | B09  | L-Proline | amino acid | 0.0394 | 5343 | 1.07E-10 |
| PM07        | B04  | Lys-Trp | dipeptide | 0.006            | 5277          | 5.29E-06 |
| PM07        | B02  | Lys-Ser | dipeptide | 0.0426           | 5105          | 1.25E-07 |
| PM03        | F05  | Cytosine  | nucleobase | 0.0361           | 4809          | 1.11E-09 |
| PM07        | F05  | Thr-Pro | dipeptide | 0.0355           | 4772          | 3.32E-06 |
| PM03        | G03  | Uric acid | nucleobase derivative | 0.0556 | 4289 | 6.27E-07 |
| PM03        | D10  | Ethylenediamine | amine | 0.0369 | 4133 | 2.78E-05 |
| PM07        | G03  | Trp-Trp | dipeptide | 0.055            | 4104          | 1.74E-03 |
| PM06        | B09  | Arg-Glu | dipeptide | 0.0404           | 4059          | 1.70E-09 |
| PM03        | F04  | Cytidine  | nucleoside | 0.0456           | 3952          | 9.78E-12 |
| PM03        | D08  | Ethylamine | amine | 0.0401 | 3799 | 8.84E-05 |
| PM06        | E11  | Gly-Pro | dipeptide | 0.0414           | 3798          | 9.96E-07 |
| PM03        | B03  | L-Histidine | amino acid | 0.0361 | 3640 | 8.27E-05 |
| PM03        | E05  | Formamide | amide | 0.0418 | 3639 | 1.96E-11 |
| PM06        | F02  | Gly-Trp | dipeptide | 0.0431           | 3632          | 1.68E-05 |
| PM03        | G09  | ε-Amino-N-Caproic acid | amino fatty acid, ε- | 0.0366 | 3389 | 4.02E-05 |
| PM03        | D06  | N-Amylamine | amine, N- | 0.0433 | 3384 | 9.99E-09 |
| PM03        | C05  | D-Aspartic acid | amino acid, D- | 0.0109 | 3334 | 3.33E-04 |
| PM03        | F10  | Uracil  | nucleobase | 0.0375           | 3204          | 2.32E-04 |
| PM03        | C10  | L-Citrulline | amino acid | 0.0377 | 3086 | 1.56E-03 |
| PM08        | C11  | Pro-Arg | dipeptide | 0.0442           | 2967          | 2.61E-03 |
| PM03        | E04  | Acetamide | amide | 0.0458 | 2926 | 7.25E-09 |
| PM03        | E07  | D,L-Lactamide | amide, DL- | 0.04 | 2859 | 1.50E-07 |
| PM03        | E03  | Tyramine | amine, Tyr derivative | 0.0442 | 2772 | 9.91E-07 |
| PM01        | F04  | D-Threonine | amino acid, D- | 0.0923 | 2699 | 1.04E-03 |
conditions that may positively or negatively affect toxin production by C. difficile or other toxigenic microorganisms. Using this approach, we measured toxin production by C. difficile ATCC 9689 under very diverse nutritional conditions, including 768 carbon, nitrogen, phosphorus, sulfur, and other nutrient sources (Table S1). Measuring hundreds of culture conditions in a quantitative and high throughput manner provides a broad perspective on toxin regulation and thus increases the probability of meaningful discoveries in toxin research. It provides another dimension beyond the effects of genetic changes.

Research has shown that toxin A and B production by C. difficile VPI 10463 is regulated by temperature through TcdR activity, the alternative sigma factor positively regulating tcdA and tcdB expression [57]. At 37°C, toxin expression was highest compared to 22 or 42°C. This temperature regulated toxin production is positively correlated to the regulation of butyric acid production, but not to other short chain fatty acids [57]. With a robust assay capability on hundreds of different PM culture conditions, this assay technology can enable simultaneously studies of toxin regulation in cells with different or same genetic backgrounds and at different temperatures. This kind of multi-dimensional analysis would greatly enrich our understanding of the bacterium, its metabolism, and environmental factors affecting regulation of toxin production.

Influences of genetic factors on toxin production, in some cases, seem very clear. For example, for some non-toxic strains examined by Hammond and Johnson, the pathogenicity locus (PaLoc) is absent and a very short fragment (127 bp) occupies the same chromosomal location [58]. Emergent in North America, Europe, and Asia since 2003 [11,18,19,20,21,22], hypervirulent strains, represented and dominated by ribotype 027 or BI/NAP1/027, produce high levels of toxin A and toxin B, which is presumably due to their harboring a tcdC repressor gene mutated at several distinct sites (an 18-bp, a 36-bp, a 39-bp deletion, or a single base pair deletion at position 117 that causes a frameshift introducing a stop codon at position 196 [18,19,47,59,60,61,62,63]).

### Table 3. Cont.

| Plate panel | Well | Chemical | Category | C. difficile Mass* | Toxin (ng/ml)b | P valuec |
|-------------|------|----------|----------|------------------|----------------|----------|
| PM01        | A03  | N-Acetyl-D-Glucosamine | acetyl amino sugar, N- | 0.0955 | 2657 | 5.17E-05 |
| PM01        | G05  | L-Alanine | amino acid | 0.113 | 2469 | 1.02E-04 |
| PM01        | F05  | Fumaric Acid | carboxylic acid | 0.018 | 2284 | 8.73E-04 |
| PM03        | E02  | β-Phenylethylamine | amine, Phe derivative, β- | 0.0448 | 2147 | 1.80E-06 |
| PM01        | G06  | Ala-Gly | dipeptide | 0.0934 | 2130 | 4.82E-07 |
| PM03        | G01  | Xanthine | nucleoside | 0.2978 | 2079 | 2.83E-05 |
| PM08        | C01  | Lys-Gly | dipeptide | 0.047 | 1950 | 1.30E-04 |
| PM08        | H03  | Gly-Gly-Gly | tripeptide | 0.0456 | 1811 | 2.93E-05 |
| PM03        | B06  | L-Lysine | amino acid | 0.0436 | 1767 | 3.75E-04 |
| PM03        | E09  | D-Galactosamine | amino sugar, D- | 0.0374 | 1723 | 3.43E-09 |
| PM08        | F03  | β-Ala-Gly | dipeptide, β- | 0.0389 | 1547 | 2.32E-07 |
| PM01        | G03  | L-Serine | amino acid | 0.0635 | 1537 | 4.48E-03 |
| PM08        | G04  | Gly-D-Asp | dipeptide | 0.0467 | 1537 | 3.45E-07 |
| PM01        | F01  | Gly-Asp | dipeptide | 0.1004 | 1507 | 3.62E-03 |
| PM03        | C07  | D-Lysine | amino acid, D- | 0.0489 | 1472 | 1.00E-04 |
| PM03        | G10  | D,L-α-Amino-Caprylic acid | amino fatty acid, DL-α | 0.0484 | 647 | 4.73E-07 |
| PM01        | F06  | Bromosuccinic Acid | carboxylic acid | 0.0335 | 615 | 8.93E-05 |
| PM01        | B01  | D-Serine | amino acid, D- | 0.1114 | 588 | 1.19E-03 |
| PM02        | G07  | L-Homoserine | amino acid, Thr isomer | 0.0071 | 587 | 7.04E-03 |
| PM03        | E10  | D-Mannosamine | amino sugar, D- | 0.0364 | 583 | 1.06E-05 |
| PM08        | G12  | D-Ala-Gly-Gly | tripeptide | 0.0538 | 515 | 1.09E-05 |
| PM02        | G10  | L-Leucine | amino acid | 0.089 | 503 | 2.97E-05 |
| PM03        | E01  | Histamine | amine, His derivative | 0.0424 | 492 | 3.79E-08 |
| PM03        | F11  | Uridine | nucleoside | 0.0373 | 484 | 2.62E-04 |
| PM03        | E08  | D-Glucosamine | amino sugar, D- | 0.0447 | 476 | 3.86E-06 |
| PM03        | A08  | L-Arginine | amino acid | 0.046 | 458 | 2.65E-05 |
| PM03        | G11  | β-Amino-N-Valeric acid | amino fatty acid, β- | 0.0424 | 435 | 5.08E-10 |
| PM1–8       | All wells | All substrates | control | 0.0517 | 50 | 3.62E-02 |
| PM1–8       | A1   | No substrate | control | 0.0517 | 44 | 9.80E-02 |

*OD (750 nm) difference between C. difficile under certain PM substrate and the same substrate without C. difficile.

*Toxin concentrations in C. difficile supernatant collected from different PM conditions, which were calculated from the average dye reduction rate by the CHO-k1 cells according to the equations in Table 1.

*The P values were obtained from t-test on the dye reduction rates of CHO-k1 cells in the presence or absence of C. difficile supernatants collected from different PM conditions.

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Table 4. PM substrates giving middle levels of toxin productions by *C. difficile* ATCC 9689.

| Plate panel | Well | Chemical                  | Category     | C. difficile Mass* | Toxin (ng/ml)† | P value* |
|-------------|------|---------------------------|--------------|-------------------|----------------|----------|
| PM07        | F06  | Trp-Ala dipeptide         |              | 0.0489            | 415            | 1.08E-04 |
| PM03        | A03  | Nitrite inorganic N-source|              | 0.0333            | 383            | 3.24E-07 |
| PM08        | B08  | Leu-Asn dipeptide         |              | 0.0482            | 381            | 2.24E-03 |
| PM06        | F07  | His-Leu dipeptide         |              | 0.0495            | 376            | 6.77E-05 |
| PM03        | F03  | Adenosine nucleoside      |              | 0.0472            | 373            | 7.02E-03 |
| PM01        | G01  | Gly-Glu dipeptide         |              | 0.0411            | 366            | 5.33E-04 |
| PM06        | E03  | Gly-Arg dipeptide         |              | 0.0403            | 352            | 6.87E-05 |
| PM08        | B12  | Lys-Asp dipeptide         |              | 0.0525            | 335            | 4.93E-05 |
| PM03        | D11  | Putrescine amine          |              | 0.0339            | 335            | 7.99E-08 |
| PM03        | G05  | Allantoin nucleobase derivative | 0.0425 | 334 | 1.48E-08 |
| PM03        | D04  | Hydroxylamine inorganic base, reducing agent | 0.0228 | 334 | 1.58E-07 |
| PM01        | D02  | D-Aspartic Acid amino acid, D-Asp | 0.0269 | 331 | 5.56E-05 |
| PM02        | B02  | N-Acetyl-Neuraminic acid acetyl amino sugar, sialic acid | 0.1163 | 328 | 1.77E-03 |
| PM04        | C12  | Cytidine 3',5'-Cyclic Monophosphate nucleotide, 3',5'-cyclic | 0.0106 | 321 | 8.83E-05 |
| PM03        | A06  | Biuret amide, carbamide derivative | 0.0394 | 307 | 3.59E-06 |
| PM01        | G10  | Methylpyruvate carboxylic acid derivative, methyl ester | 0.0795 | 299 | 5.31E-05 |
| PM08        | H08  | Gly-Phe-Phe tripeptide   |              | 0.2751            | 298            | 4.18E-07 |
| PM08        | G05  | Gly-D-Ser dipeptide      |              | 0.0543            | 297            | 8.49E-06 |
| PM08        | H07  | Val-Tyr-Val tripeptide  |              | 0.0382            | 296            | 2.62E-06 |
| PM03        | E06  | Glucuronamide sugar acid, amide (6c) | 0.0369 | 280 | 1.10E-06 |
| PM07        | A08  | Lys-Glu dipeptide        |              | 0.0425            | 271            | 1.01E-04 |
| PM01        | G04  | L-Threonine amino acid, Thr | 0.0643 | 243 | 3.70E-03 |
| PM07        | H05  | Val-Asp dipeptide        |              | 0.0469            | 225            | 1.12E-04 |
| PM03        | C02  | L-Valine amino acid, Val  |              | 0.0431            | 223            | 2.01E-04 |
| PM08        | H05  | Gly-Gly-Leu tripeptide  |              | 0.0305            | 215            | 1.03E-04 |
| PM02        | B08  | Arbutin sugar hydroquinone (12c), glycoside | 0.128 | 213 | 5.36E-05 |
| PM03        | C04  | D-Asparagine amino acid, D-Apn | 0.0321 | 196 | 2.31E-04 |
| PM07        | E04  | Ser-Leu dipeptide        |              | 0.0404            | 187            | 3.43E-04 |
| PM07        | G05  | Tyr-Ala dipeptide        |              | 0.0331            | 186            | 5.79E-05 |
| PM03        | H10  | Gly-Glu dipeptide        |              | 0.0472            | 184            | 5.43E-03 |
| PM08        | E03  | Trp-Val dipeptide        |              | 0.0276            | 184            | 1.26E-04 |
| PM08        | E12  | Val-Pro dipeptide        |              | 0.0425            | 183            | 2.01E-04 |
| PM06        | F12  | His-Trp dipeptide        |              | 0.0442            | 178            | 1.49E-04 |
| PM08        | E08  | Val-Glu dipeptide        |              | 0.0481            | 178            | 1.11E-05 |
| PM07        | H02  | Tyr-Tyr dipeptide        |              | 0.0415            | 174            | 2.16E-04 |
| PM01        | H12  | 2-Aminoethanol amine, alcohol, 2- | 0.0853 | 173 | 1.15E-03 |
| PM03        | C01  | L-Tyrosine amino acid, Tyr | 0.1528 | 172 | 1.52E-05 |
| PM03        | C03  | D-Alanine amino acid, D-Ala | 0.038 | 171 | 1.70E-04 |
| PM03        | B12  | L-Tryptophan amino acid, Trp | 0.0299 | 165 | 2.91E-04 |
| PM02        | E01  | Capric acid fatty acid  |              | 0.014             | 162            | 3.24E-04 |
| PM02        | A06  | Dextrin sugar, polysaccharide | 0.0478 | 162 | 3.99E-03 |
| PM08        | G06  | Gly-D-Thr dipeptide      |              | 0.0549            | 157            | 3.21E-05 |
| PM03        | E12  | N-Acetyl-D-Galactosamine acetyl amino sugar, N- | 0.0355 | 153 | 3.06E-04 |
| PM07        | A11  | Lys-Lys dipeptide        |              | 0.0427            | 150            | 5.07E-05 |
| PM03        | C12  | L-Orotate amino acid, Orn | 0.0369 | 141 | 3.49E-04 |
| PM02        | D02  | Salicin sugar phenol, (β-glucoside (13c)0.1291 | 140 | 3.61E-03 |
| PM02        | C04  | D-Lyxose sugar (5c), D- | 0.1508 | 139 | 3.82E-03 |
Table 4. Cont.

| Plate panel | Well Chemical                     | Category         | C. difficile Massa | Toxin (ng/ml)bP valuec |
|-------------|-----------------------------------|------------------|-------------------|------------------------|
| PM03        | D02 N-Pthaloyl-L-Glutamic acid    | amino acid derivative | 0.0344           | 137                    | 4.69E-05               |
| PM02        | H04 L-Valine                      | amino acid, Val   | 0.0917            | 135                    | 1.28E-02               |
| PM07        | G04 Trp-Tyr                       | dipeptide        | 0.0451            | 134                    | 5.89E-05               |
| PM02        | E02 Caproic acid                  | fatty acid       | 0.0568            | 133                    | 1.90E-03               |
| PM02        | G12 L-Methionine                  | amino acid, Met   | 0.0818            | 127                    | 6.01E-04               |
| PM06        | G04 Ile-Arg                       | dipeptide        | 0.0442            | 122                    | 3.82E-03               |
| PM03        | A01 Negative Control              | negative control | 0.0403            | 122                    | 1.90E-04               |
| PM03        | B04 L-Isoleucine                  | amino acid, Ile  | 0.0563            | 122                    | 9.75E-04               |
| PM02        | D06 D-Tagatose                    | sugar (6c), D-    | 0.138             | 121                    | 8.44E-04               |
| PM03        | C11 L-Homoserine                  | amino acid, Thr isomer | 0.0293         | 119                    | 2.52E-05               |
| PM01        | A09 D-Alanine                     | amino acid, D-Ala | 0.1233            | 118                    | 9.72E-06               |
| PM07        | A03 Leu-Ser                       | dipeptide        | 0.037             | 116                    | 1.51E-04               |
| PM03        | C08 D-Serine                      | amino acid, D-Ser | 0.0465            | 110                    | 7.29E-05               |
| PM03        | A11 L-Cysteine                    | amino acid, Cys   | 0.0394            | 108                    | 6.64E-05               |
| PM03        | D12 Agmatine                      | amine, Arg derivative | 0.043            | 108                    | 3.06E-04               |
| PM01        | C07 D-Fructose                    | sugar (6c), D-    | 0.1435            | 106                    | 1.34E-06               |
| PM01        | H08 Pyruvic Acid                  | carboxylic acid, α-keto acid | 0.093          | 103                    | 5.67E-04               |
| PM02        | H02 L-Phenylalanine               | amino acid, Phe   | 0.0714            | 103                    | 2.46E-02               |
| PM1–8       | All wells All substrates          | control          | 0.0517            | 50                     | 3.62E-02               |
| PM1–8       | A1 No substrate                  | control          | 0.0517            | 44                     | 9.80E-02               |

OD (750 nm) difference between C. difficile under certain PM substrate and the same substrate without C. difficile.

Toxin concentrations in C. difficile supernatant collected from different PM conditions, which were calculated from the average dye reduction rate by the CHO-k1 cells according to the equations in Table 1.

*The P values were obtained from t-test on the dye reduction rates of CHO-k1 cells in the presence or absence of C. difficile supernatants collected from different PM conditions.

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some conflicting evidence has shown that truncation of the repressor gene tdIC does not cause higher toxin production in ribotype 027-related strains [64]. In addition, other groups have reported a lack of association of tdC mutation type with disease severity in toxigenic C. difficile [65,66,67,68,69]. Therefore, regulation by genetic factors may be more complicated than expected. A comparable and direct functional or phenotypic toxin assay, such as the assay that we have demonstrated, should be helpful in sorting this out.

Spo0A, the master regulator for sporulation initiation, positively regulates toxin production [70]; and SigH, the key phase transcriptional factor, negatively regulates the toxin expression [71]. CodY, a global regulator of gene expression, directly binds to the promoter of tdCR with high affinity, down-regulating toxin genes tcdA and tcdB [72]. This binding is enhanced by GTP and branched-chain amino acids (leucine, isoleucine, and valine). Therefore, CodY may integrate toxin production with the nutrient status of C. difficile. [72,73]. In our studies with the type strain of C. difficile, ATCC 9689, we found that toxin B production was most strongly repressed by some dipeptides containing leucine and the triple-leucine tripeptide as nitrogen sources in the presence of glucose. This appears to agree with the observation of leucine’s enhancement of down-regulation via CodY. Leucine peptides may be taken up more efficiently than leucine. It would be very interesting to quantitatively measure functional toxin production with isogenic mutants of relevant regulatory genes under hundreds of nutritional culture conditions. This could provide new insights into understanding the coordination of nutrient metabolism and toxin production by these regulatory genes. Furthermore, if the leucine peptides are broadly and rapidly active in suppressing toxin production, they could perhaps be utilized to quell C. difficile induced toxicity in patients.

Over the past decades, there have been some prior studies of the effects of nutritional factors on C. difficile toxin production. It is well known that biotin deficiency increases toxin A and toxin B production significantly in some strains [74,75] whereas glucose has been shown to reduce toxin production [76,77,78,79]. In our studies, we used vitamin sufficient defined media by adding 0.5× of a vitamin mix (RPMI1640 vitamins, Sigma, used for mammalian cell culture). For biotin, the concentration used for C. difficile culture was 410 nM. Therefore, in these studies, biotin deficiency was not a factor. Looking at diverse carbon sources, we confirmed that glucose gives relatively low toxin production. Compared to N-Acetyl-D-Glucosamine (2657.19 ng/ml, PM1 A3), glucose gave a much lower level of toxin production (78.86 ng/ml, PM1 C9), only slightly higher than that of no substrate control of the panel (57.83 ng/ml, PM1 A1). The inhibition of C. difficile toxin production by glucose may be due to carbon catabolite repression through catabolite control protein A, CcpA [78]. On the other hand, glucose limitation reduced toxin production dramatically [74,75,77], which was also true in our observations when C. difficile was inoculated in PM3 with reduced glucose as carbon source (data not shown).

An influence of arginine on C. difficile toxin production has been noted previously, but the effect has not been consistent in reports: (1) toxin production increases with arginine addition in complex medium [80]; (2) toxin production increases with the absence or insufficiency of arginine [81]. In our assay, some arginine...
and amines were strong toxin inducers in a plant pathogenic fungus using PM panels [5], where arginine dipeptides agrees with results of Osgood et al [80] and results from Table 3). This increase in toxin production by arginine or arginine dipeptides increased toxin production dramatically (Figures 4A, 4C, Table 3), with less increase by arginine (458 ng/ml, Figure 4C, which are even higher (Figures 4A, 4C, Table 3, S2). This agrees with the results of Karasawa et al [81] who found that arginine is not absolutely required for increased toxin production. Not surprisingly, the regulation appears to be multifactorial. For example, arginine dipeptides containing branched-chain amino acids (Leu, Ile, and Val), gave much lower levels of toxin production than other arginine dipeptides, even lower than that of arginine itself. For example, C. difficile ATCC 9689 produced dipeptides increased toxin production dramatically (Figures 4A, 4C, Table 3). This increase in toxin production by arginine or arginine dipeptides agrees with results of Osgood et al [80] and results from a plant pathogenic fungus using PM panels [5], where arginine and amines were strong toxin inducers in Fusarium graminearum. Culture conditions with nitrogen sources other than arginine or arginine dipeptides, can also produce high toxin levels, some of which are even higher (Figures 4A, 4C, Table 3, S2). This agrees with the results of Karasawa et al [81] who found that arginine is not absolutely required for increased toxin production. Not surprisingly, the regulation appears to be multifactorial. For example, arginine dipeptides containing branched-chain amino acids (Leu, Ile, and Val), gave much lower levels of toxin production than other arginine dipeptides, even lower than that of arginine itself. For example, C. difficile ATCC 9689 produced
282 ng/ml on Arg-Ile; 122 on Ile-Arg, 67 on Leu-Arg, 54 on Arg-Leu, 36 on Val-Arg, and 32 on Arg-Val (Table S2). These low toxin production levels could be explained, at least in part, by the branched-chain amino acids enhancing CodY’s ability to down-regulate tcdA and tcdB [72]. Other interesting observations may have different underlying explanations. For example, while Arg-Ala and Lys-Arg gave very high toxin production, 10,080 and 5536 ng/ml, respectively (Figure 4A, Table 3), Ala-Arg gave a strikingly low level toxin production of 34 ng/ml (Table S2) and Arg-Lys gave a middle level of 308 ng/ml (Table S2). Presumably the specificity of toxin induction reflects differences in the transport and hydrolysis of the specific peptides by C. difficile.

To our knowledge, this is the first report that nucleobase and nucleoside biochemicals are among the strongest C. difficile toxin inducers. In particular, adenine and guanosine top all substrates measured in this study, giving very high levels of toxin production (>168,000 ng/ml and 10402 ng/ml, respectively) when they serve as nitrogen source in the presence of 5 mM glucose. In this nucleobase and nucleoside substrate group, many derivatives of purines and pyrimidines can stimulate high C. difficile toxin production as well (Figure 4A, Table 3). The reason is not known, but it is clearly not a random phenomenon. Maegawa et al [82] report that there is a linkage between toxin production and purine biosynthesis in C. difficile. Further investigation of these nucleobases and nucleosides inducing high levels of toxin may provide a new insight into the biochemical and molecular basis of C. difficile toxin regulation and may also help in explaining the clinical severity of CDI. Given induction of high toxin production by uric acid, a metabolite of purine, a question could be rationally asked: what is the correlation between circulating level of uric acid and severity or even mortality of CDI in patients?

Figure 4. Examples of high toxin-inducing PM substrates for C. difficile ATCC 9689. A. Top toxin inducers. Note that adenine as a nitrogen source is the strongest toxin inducer of all tested PM substrates in this study. g-D-Glu-Gly = γ-D-Glu-Gly; β-Ala-His = β-Ala-His; γ-Aminobutyric = γ-Aminobutyric acid. B. High toxin production induced by some carbon sources in PM1 and 2. C. High toxin production induced by some amino acids as nitrogen sources. Note that L-proline is the highest toxin inducer in the amino acid category.
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The nutrients in the basal medium used in this study were present at low concentrations and systematically varied. Each well of panels PM3, 6, 7, and 8 contains these same components except one nitrogen source which differs from well to well (Materials & Methods, Table S1). So, the differences in toxin production are truly related to the specific difference of the nitrogen source among the wells. This is in contrast with previous studies where much richer and more complex media were typically used. For example, amino acids were added up to 35 mM plus 27.78 mM glucose and vitamins and minerals [83], or 39 mM amino acids plus 11.11 mM glucose and other nutrients [84], or 82 mM amino acids plus 11.11 mM glucose and other nutrients [74]. BHI plus 20.83 mM glucose (0.375%) or TV (3% bacto tryptose and 2% yeast extract) plus 55.5 mM glucose were used [72]; PT (peptone-yeast extract) plus 50 mM glucose (PT_0G) or PT plus 10 mM cysteine (PY_10) were reported [76]. In our study, the relatively low nutritional PM culture conditions may partially contribute to the high toxin production, which agrees well with the notion that the toxin production increases when the bacteria are under stresses such as environmental and nutritional factors. In fact, nutrients in the colon are at low levels based on human feces analysis [85] and *Clostridium difficile* must compete for nutrients against other flora [86, 87, 88]. According to Goldblith’s analysis [85], vitamins account on the average for about 0.01% of the feces in human and biotin is about 0.133 mg in feces collected over a period of 24 h (≈544 nM, given 0.133 mg biotin collected and assuming 1000 g wet weight of feces over 24 h). Therefore, the levels of nutrients of our PM culture conditions seem more close to the natural nutrient levels in the colon.

It is reported that before antibiotic treatment the average toxin B concentration in CDI patients stools was 58.29 pM (≈15.68 ng/ml) [90]. After antibiotic treatment the toxin B level was 0.89 ng/ml [89]. Human colonic mucosae ex vivo testing showed damaging effects after 5 h of exposure to toxin B at a concentration about 0.2 nM (53.8 ng/ml) [90]. The TcdBHV toxin can cause broad tissue damage to zebrafish embryos at concentrations about 1 nM (≈270 ng/ml) and is cytotoxic to CHO-k1 cells with TCD50 of 23.7 pM (≈6.4 ng/ml) [44]. In this study, the lowest concentration of *Clostridium difficile* toxin B that can be reliably detected and quantified is as low as 0.45 pM (≈0.122 ng/ml). Therefore, our method could find applications in direct measurement of clinical samples from patients or animals to improve diagnosis, treatment, and epidemiologic studies of CDI.

*Clostridium difficile* strain VPI 10463 (ATCC 43255) is a high toxin producer [91] and is the toxinoype reference strain, classified as toxinoype O [92, 93]. Strain ATCC 9689 is the *Clostridium difficile* type strain, sharing the same toxinoype with VPI 10463. Presumably the toxins produced by the two strains are similar. Indeed, the neutralizing antibody raised against toxin B protein from VPI 16403 (Gallus Immunotech) is highly effective in neutralizing toxins from both strains. In a comparison study, we examined toxin production in the bacterial supernatants of the two strains under identical PM culture conditions. From this we estimate that VPI 10463 produced ~100 to ~1000 times more toxin in quantity (data not shown). However, some of the highest toxin-producing substrates (e.g., adenine and guanosine) already stimulate very high toxin production by ATCC 9689 and a further induction of 100–1000 fold in VPI 10463 seems less likely. A more likely explanation is that the toxin B molecules produced by VPI 10463 are more potent than those of ATCC 9689.

The toxins A and B we purchased (Listlab) have been subjected to purification and lyophilization processes whereas the toxin produced in this study are crude toxin protein preparations that are just filtered to remove cells and then assayed quickly. Given the large size of toxins A and B (300 kDa and 270 kDa, respectively), it is possible that the purified proteins may suffer some damage or conformational changes by physical or chemical forces during the processes of purification and lyophilization.
Crude toxin proteins tested immediately or within a very short period of time without freezing may be more intact and therefore may retain more functionality and potency. The intactness of the toxin molecules may be particularly important for toxin autoprocessing for the toxin to become cytotoxic. If the toxin molecules in a sample are less damaged than the standard purified toxins against which the sample toxin is quantified, it may lead to assigning an artificially high toxin concentration to the sample.

Our PM culture conditions usually produce high level (or high potency) toxin at very low C. difficile mass, suggesting that we are more likely to be overestimating the toxin level as our preparations may contain higher levels of intact toxin molecules.

To generalize this assay technology, we also tested some other species of clostridia and other bacterial genera that are known or potential producers of cytotoxins. Species tested in this study that demonstrated cytotoxicity include C. perfringens, C. tetani, C. sordellii, Bacillus cereus, Escherichia coli O157:H7, Shigella dysenteriae, and Listeria monocytogenes. Species tested that did not demonstrate cytotoxicity include E. coli BW30270, S. sonnei, L. innocua. Thus, our new cytotoxicity assay has been further validated with diverse bacterial species.

Figure 6. Cell-based cytotoxicity assay of other bacteria. A. Cytotoxin assay comparison of Clostridial species. Five μl of bacterial supernatant from each well of the PM of each Clostridial species, C. difficile (ATCC 9689), C. perfringens (ATCC 25763), and C. sordellii (ATCC 9714), were used in this assay. CHO-k1 is the indicator cell in this dye reduction assay. Upper panel: supernatants from PM1; Lower panel: supernatants from PM6. In the lower panel, the wells above the lines contain arginine dipeptides as the nitrogen source. B. Aerobic bacterial toxin assays and comparison of supernatants from PM1 between toxigenic and non toxigenic strains or species. Vero is the indicator cell for E. coli strains and Shigella species. CHO-k1 is the indicator cell for Listeria species. Upper panel: toxigenic strains or species; Lower panel: non-toxigenic strains or species. For all: darker color indicates higher dye reduction rate and less toxin produced in that well; lighter color indicates lower dye reduction rate and more toxin produced in that well. doi:10.1371/journal.pone.0056545.g006
In addition to studying the regulation of toxin production in species and strains that are known producers, the sensitive and general nature of this approach will also enable the discovery of novel toxin-producing strains and novel toxins, especially toxins that are produced only under a very restricted set of culture conditions, as was the case with the *F. graminearum* toxin [6] which inspired these studies.

In summary, this is the first demonstration of simultaneous study of a large number of culture conditions influencing bacterial toxin production in diverse bacteria. Toxin production is a central issue in pathogenesis of *C. difficile* and other pathogenic...
microorganisms. Reliable, quantitative, sensitive, and robust assays for functional toxins are essential and critical in scientific research and clinical practice. The functional toxin assay method presented here is such an assay technology. It is based on the gold standard cell-based cytotoxicity assay and also takes advantage of PM technology. With hundreds to thousands different culture conditions, this toxin assay technology can provide a wealth of information and insights into the regulation of the toxin production and pathogenesis of toxigenic microorganisms. It may also find beneficial applications in clinical and epidemiological research.

Materials and Methods

Phenotype MicroArray (PM) panels, chemicals, toxins, anti-toxins, bacterial strains, and mammalian cells lines

PM panels, inoculating fluid IF-0a GN/GP Base (or IF-0a for short), Redox Dye Mix MB and inoculating fluid IF-M1 are from Biolog, Inc. (Hayward, CA, USA). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. Yeast extract (YE) was from Oxoid (UK). Clostridium difficile toxin A and toxin B were from List Biological Laboratory (Listlab, Campbell, CA, USA). It is important to use antibody of high quality and antibody from some vendors was not satisfactory. We ultimately sourced anti-toxin A and anti-toxin B polyclonal chicken IgYs from Gallus Immunotech (Fergus, Ontario, Canada). Clostridium difficile strains: type strain ATCC 9689 and Toxinotype 0 strain VPI 10463 (ATCC 43255) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Some other bacteria obtained through commercial sources include: C. perfringens (ATCC 25763), C. tetani (ATCC 19406), C. sordellii (ATCC 9714), Bacillus cereus (ATCC 14579), Escherichia coli O157:H7 (ATCC 43894), Shigella dysenteriae (ATCC 11835), S. sonnei (ATCC 25931), L. innocua (ATCC 33090)

Others were obtained as gifts, including E. coli BW20270 (from Barry Wanner, USA), Listeria monocytogenes strains P14 and P14-A (from Jose Vasquez-Boland, UK). Mammalian cell lines CHO-k1, Vero, HT-29, and A549 were also purchased from ATCC. C. difficile toxin production in a 96-well panel format. This process is applied to all anaerobic bacteria. The incubation time for different bacteria may vary before toxins are collected. To collect toxins from aerobic bacteria, the process is the same as that for anaerobic bacteria except bacteria are incubated in an aerobic incubator instead of an anaerobic chamber.

Bacterial preculture and inoculum preparation

C. difficile and other anaerobic bacterial strains were routinely pre-cultured on BUA+B agar (Biolog) inside an anaerobic chamber (Bactron IV) at 36°C with a gas atmosphere of 5% H2, 3% CO2, and 90% N2. C. difficile fresh cultures (20–24 h) on BUA+B were used for the PM panel inoculation. The inoculum of C. difficile was from fresh cultures in late log phase (Figure S2) which were examined and found to be free of endospores (Figure S3). Inocula were conveniently prepared by removing colonies from a BUA+B agar plate using a swab, and resuspending in IF-0a inoculating fluid. The bacterial suspension was adjusted in IF-0a to achieve a 40% transmittance (T40) using a Biolog Turbidimeter, which was measured spectrophotometrically as 0.139±0.002 O.D. (at 750 nm in a Multiskan Ascent). The suspension was further diluted 1:16 in IF-0a. This cell density was directly used for PM panel inoculation. This inoculum (T40 1:16) when plated on BUA+B gave a count of 9.07±0.07 CFUs/ml.

PM panel special pretreatment for anaerobic bacteria

Prior to their use with anaerobic bacteria, all PM panels were converted to an anaerobic state by thorough deoxygenation. To do this, the PM panel packaging bags are cut to open one end. Two oxygen absorbers (Ageless sachets, Mitsubishi) are inserted into the bag along with the original desiccate sachet and then the bag is resealed with a heat sealer. If the bag has a good seal, the sachets will absorb the air in the bag so that the packaging appears as if it is shrink-wrapped. This operation can be done on the lab bench. The resealed PM panels can be kept at room temperature for 1 day and then stored in the refrigerator (2–8°C) for additional days or months before use. The thoroughly deoxygenated panels can then be warmed up to room temperature before being used in an experiment. It is critical that the panels are resealed well, which allows the Ageless sachets to completely remove oxygen from the panels. It is not recommended to deoxygenate the PM panels by putting them in the anaerobic chamber because the moisture inside the chamber may destabilize some substrates. To deoxygenate Biolog IF-0a GN/GP Base, the bottle caps were loosened and the bottles were placed inside the anaerobic chamber for 3 days before use.

PM media and bacterial toxin collection

PM panels are 96-well microplates containing a different substrate in each well. PM1 and PM2 are carbon source panels. PM3, 6, 7, and 8 are nitrogen source panels. PM4 contains various phosphate and sulfur sources; PM5 contains various biosynthesis pathway endproducts and nutrient supplements. The substrates in the PM wells are shown in Table S1. In addition to a unique substrate, each well of these metabolic panels also contains the needed minimal medium components [2] but without ferric chloride, tetrazolium violet, and sodium pyruvate added.

To produce and collect bacterial culture supernatants containing toxins, C. difficile or other bacterial species were inoculated in duplicate or triplicate into panels PM1–8 at T40 1:16 and incubated for an optimum length of time, e.g., 3 days for C. difficile. The inoculating fluid for C. difficile consists of Biolog IF-0a GN/GP Base, 0.5 × RPMI 1640 vitamins, and 0.2% yeast extract (sterilized by filtration) for PM1, 2 or 0.05% yeast extract for PM3, 4, 5, 6, 7, and 8. To provide the carbon source in PM3–8, glucose at a final concentration of 5 mM was added to the inoculating fluid. To obtain a crude toxin preparation free of cells, the bacterial liquid cultures were transferred to a 96-well filter plate ( Pall) and filtered by centrifugation at 2000 rpm for 3 minutes (Hermle Z 360 K, rotor model C-0360-50). The filtrates were used immediately (the same day) or kept in a sterile 96-well plate (Biolog) sealed with tape for brief storage at 4°C before use. No bacteria controls (i.e. no toxin controls, or PM substrate controls) were used. PM substrate solutions were rehydrated from the corresponding PM panels, obtained by following the same procedure for bacterial supernatants described above except no bacteria inoculated into the PM panels. All experiments described above were done multiple times.
(2–4) as independent replicates (2–3) and each was followed by cytotoxicity assays on the bacterial filtrates collected.

All aerobic bacteria tested in this study were handled on the bench and grown on Biolog agar medium (BUG+B) as a preculture and then inoculated and incubated aerobically in PM panels without shaking. After 24 h of incubation in the panels at 37°C, the bacterial supernatants were collected using the 96-well filter plates and centrifugation, and then handled and tested as described above.

The toxin-containing supernatants should be used as soon as possible after harvest because refrigeration temperature does not completely maintain the toxins during long term storage and the potency of the toxins was observed to decrease over time. Better long term storage of the toxins requires purification and lyophilization. These same procedures were followed for preparing PM substrate suspension controls without C. difficile.

Determination of bacterial mass in wells of PM panels

To determine bacterial mass kinetics, PM panels inoculated with C. difficile in triplicate were removed from the anaerobic chamber at 24, 48, or 72 h of incubation. Bacterial mass was determined by measuring the optical density of wells at 750 nm using a microplate reader (Multiskan Ascent). Compared to 48 h or 72 h, the values of OD-750 at 24 h of incubation were the highest under most PM well conditions. With a few exceptions, all the OD-750 values decreased more or less at 48 h and further decreased at 72 h (Figure S1). Given the nutritional limitations of the media, the growth was limited, but the kinetic trends were clear and similar to each other among the various culture conditions (Figure S1).

To determine the bacterial mass at the end point and before harvesting toxin-containing supernatants, the PM panels with C. difficile in replicates were removed from the anaerobic chamber at 72 h and the mass was determined as described above. To obtain net bacterial mass, the OD-750 value from the corresponding well of an uninoculated plate is subtracted from the OD-750 value of the inoculated well. These bacterial net mass values can be used to normalize toxin production when a specific cell productivity measurement is needed.

Mammalian cell lines and their cytotoxicity and neutralization assays

Cell lines CHO-k1, Vero, HT-29, and A549 were grown at 37°C in an incubator atmosphere of 5% CO₂ in standard T75 cell culture flasks with RPMI 1640 medium plus 10% FBS and 1 × Pen/Strep, without phenol red. The cells were allowed to grow for 20–24 h before harvesting for experiments. This young cell culture was then prepared in an assay medium (Biolog IF-MI inoculating fluid plus 2 mM glucose, 2 mM glutamine, 2% FBS, and 1 × Pen/Strep) at a density of 200,000 cells per ml. A 100 µl aliquot of this cell suspension was plated into each well of tissue culture treated 96-well microplates, the assay plate. Five microliters of toxin preparation either from serial titrations of purified standard toxin (Listlab) or from supernatants of microbial culture filtrates collected from each PM culture condition were transferred to each well of the assay plate immediately following the cell plating. This makes the toxin preparation a 21-fold dilution [5+100]/5]. Five µl of corresponding PM substrate solution were used as no toxin control. The assay plates of the treated indicator cells were then incubated at 37°C with 5% CO₂ for 18–20 h.

For neutralization experiments, a neutralizing antibody was mixed with the indicator cell suspension immediately prior to cell plating. The cell plating was followed immediately by addition of the toxin preparation to the plated cells. After 18–20 h of incubation, cell morphologies were observed under the microscope (10×10 magnification) and images were recorded with a digital camera. These same cells were further tested for their ability to reduce the Biolog Dye Mix MB. Twenty µl of the dye solution was transferred to each well. The plates were then placed into the OmniLog PM instrument (Biolog, Inc.) for incubation and kinetic data collection for 3 h or longer. As the dye is reduced, a purple color is irreversibly developed. The healthier the cells are, the more NADH they produce and the higher the rate of dye reduction [4].

Determination of dye reduction rate by the mammalian cells

The dye reduction by indicator cells was kinetically measured during the incubation period by the OmniLog instrument. The resulting dye reduction values were imported into a PM analysis program, which can calculate a rate of dye reduction based on a linear regression algorithm. The rate of dye reduction within the first few hours of incubation (e.g., 0 through 3 h) was calculated. The effects of standard C. difficile toxin B or C. difficile supernatants collected from different PM conditions on the rate were studied. The average rates of the standard toxin or of the supernatants were used in all subsequent calculations.

To correct for the influence (positive or negative) of a given PM substrate on the rate, the dye reduction rate of the PM substrate control is measured. A ratio of a PM substrate is derived from the control set and defined as a quotient of the average rate of all wells for a given PM panel and its replicates (e.g., an average of 3 PMI panels is obtained from 3 × 96 wells) divided by the average of a given well in that panel. So, if a PM substrate has a positive influence on the dye reduction rate of the indicator cell line, the ratio will be smaller than 1, otherwise greater than 1. For example, D-glucose (Table S1, PMI C9) could positively influence dye reduction because the substrate control experiments showed it was associated with higher dye reduction rate than the panel average (78.48 vs 74.93). So, the ratio for D-glucose is smaller than 1 (0.9547). The average dye reduction rate of CHO-k1 in the presence of C. difficile supernatant from D-glucose is 57.19, but the corrected dye reduction rate is 57.19 × 0.9547 = 54.6.

In general, the rate of dye reduction by cells can be affected by many factors, e.g., cell lines, cell number and fitness, adverse environmental conditions (e.g., toxic chemicals), nutrient or energy source used to support cell respiration, the type of redox dyes, and so on. In these experiments, however, all factors and conditions given are controlled except the levels of toxins produced by the microorganism under the different culture conditions in the PM panels.

As mentioned above, all experiments were done multiple times and as independent replicates. From these, the averages and standard deviations of dye reduction rates were calculated, which are expressed as a mean +/− standard deviation and plotted as histograms exemplified by Figure 3D. Statistical analyses were performed using Microsoft Office Excel TTEST via Excel automation on the rates between the experimental set and the control set on a well-by-well basis. The data were treated as two-tailed distributions with unequal variance. The difference is considered significant only if the probability of no difference between the means of the two sets is smaller than 5%, P<0.05.

Having measured the effect of the toxin as a dye reduction rate, the next step is to convert the rate into a concentration value for the toxin.
Determination of toxin production by *C. difficile* and other Clostridium species

Anaerobic bacteria used in this study including *C. difficile*, *C. perfringens*, *C. tetani*, and *C. sordellii*, were incubated for three days before removal from the anaerobic chamber for cell mass measurement and toxin collection. (1) Establishment of standard curves of toxin and dye reduction rate. *C. difficile* toxins A and B from Listlab were used as standards to determine the relationship between toxin concentration and the corresponding level of inhibition of dye reduction rate by the indicator mammalian cells. Cytotoxicity and dye reduction assays with serial 2-fold or 3-fold standard toxin titrations were performed multiple times. For a given indicator cell line, the known concentrations of the toxin (ng/ml) used in the assay were plotted on the Y-axis with a log10 scale against corresponding dye reduction rates plotted on the X-axis (Figure S4). Using Microsoft Office Excel 2002 software, the plots were fitted to curves by non-linear regression analyses and the equations for predicting concentrations from the rates were generated (Figure S4, Table 1). (2) Determination of toxin production by *C. difficile* under different PM culture conditions. The corrected average dye reduction rates from the *C. difficile* supernatants were used to calculate the toxin concentration according to the equations (Figure S4, Table 1). The calculations were performed robustly by employing Excel worksheet functions.

Supporting Information

Figure S1 *C. difficile* mass kinetics in wells of PM panels. The bacterial mass was determined at 24, 48, and 72 h of incubation as described in Materials and Methods. A, PM1. B, PM3. (TIF)

Figure S2 Growth curve of *C. difficile* colonies on BUAb. Single colonies of *C. difficile* (ATCC 9689) grown on BUAb were picked up and cell counts were made at the time points, 18, 24, 48, and 72 h. As the colony size was very small at 18 h, 4 colonies were used instead of one, but the count of colony forming units (CFUs) was normalized to a single colony by dividing by 4. To count the viable cells, the colonies were suspended in IF-0a followed by serial dilution and plating on BUAb+B. The number of viable cells was determined by counting CFUs. (TIF)

Figure S3 Endospore formation of *C. difficile* (ATCC 9689) at different ages on BUAb+B. At 24, 48, or 72 h of incubation on BUAb+B, a cell suspension was made from a single colony in 1 ml IF-0a inoculating fluid. The cell suspension was then incubated in an eppendorf tube without or with 200 proof ethanol (cell suspension : ethanol = 1:1) for 1 h inside the anaerobic chamber. Then, 30 μl of each suspension was plated on a BUAb+B plate. All plates were incubated in the chamber at 36°C for 24 h and then photographed. As shown, without ethanol, a bacterial lawn was formed from cultures of different ages on BUAb+B (24, 48, and 72 h). Endospores appear as colonies that survive the ethanol treatment. Note that no endospores were formed from the 24 h culture but then the number of endospores increased as the culture age increased (72 h>40 h). (TIF)

Figure S4 Plots of dye reduction rate by CHO-k1 cells against corresponding standard *C. difficile* toxin B. A serial 3-fold titrations of standard toxin B (Listlab) was performed on CHO-k1 cells. From top panel to bottom: The toxin concentrations decreased. Dye reduction kinetics were recorded by the OmniLog instrument and reduction rates (0–3 h) at different toxin concentrations were calculated using PM Analysis Software. The known standard toxin concentrations (Y-axis) were plotted against the corresponding Mean ± Stdev of the rates (X-axis). Regression analyses on the data were performed using Microsoft Excel 2002 software. Each equation given was suitable only for a certain range of toxin level as illustrated, and may be suitable only for the given conditions (cell line, assay conditions, type of reducible dye, etc.) as described in the text. (TIF)

Table S1 Plate maps of Phenotype MicroArrays microplate panels PM1 through PM8. (PDF)

Table S2 Toxin production of *C. difficile* ATCC 9689 under different PM conditions. (PDF)

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Author Contributions

Conceived and designed the experiments: XHL BB. Performed the experiments: XHL BB. Analyzed the data: XHL BB. Contributed reagents/materials/analysis tools: XHL. Wrote the paper: XHL BB.

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