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# Tetanus toxin production is triggered by the transition from amino acid consumption to peptides

Cuauhtemoc Licona-Cassani [a, c, 1], Jennifer A. Steen [a, 1], Nicolas E. Zaragoza [a], Glenn Moonen [b], George Moutafis [b], Mark P. Hodson [a, d], John Power [b], Lars K. Nielsen [a], Esteban Marcellin [a, *]

[a] Australian Institute for Bioengineering and Nanotechnology (AIBN), The University of Queensland, Brisbane, QLD 4072, Australia
[b] Zoetis, 45 Poplar Road, Parkville, VIC 3052, Australia
[c] National Laboratory of Genomics for Biodiversity (LANGEBIO), Cinvestav-IPN, Irapuato, Mexico
[d] Metabolomics Australia Queensland Node, AIBN, The University of Queensland, Brisbane, QLD 4072, Australia

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ABSTRACT

Bacteria produce some of the most potent biomolecules known, of which many cause serious diseases such as tetanus. For prevention, billions of people and countless animals are immunised with the highly effective vaccine, industrially produced by large-scale fermentation. However, toxin production is often hampered by low yields and batch-to-batch variability. Improved productivity has been constrained by a lack of understanding of the molecular mechanisms controlling toxin production. Here we have developed a reproducible experimental framework for screening phenotypic determinants in *Clostridium tetani* under a process that mimics an industrial setting. We show that amino acid depletion induces production of the tetanus toxin. Using time-course transcriptomics and extracellular metabolomics to generate a 'fermentation atlas' that ascribe growth behaviour, nutrient consumption and gene expression to the fermentation phases, we found a subset of preferred amino acids. Exponential growth is characterised by the consumption of those amino acids followed by a slower exponential growth phase where peptides are consumed, and toxin is produced. The results aim at assisting in fermentation medium design towards the improvement of vaccine production yields and reproducibility. In conclusion, our work not only provides deep fermentation dynamics but represents the foundation for bioprocess design based on *C. tetani* physiological behaviour under industrial settings.



## 1. Introduction

*Clostridium tetani*, the causative agent of tetanus, is an important pathogen of humans and animals. The disease occurs when a wound is infected with vegetative cells or spores which then produce an extracellular neurotoxin (tetanospasmin or TeNT) that blocks inhibitory neurotransmitters in the central nervous system [1]. To date, much of the research on *C. tetani* has been focussed on the mechanism of TeNT [2–6]. More extensive research has been restricted by the absence of reproducible culturing conditions and genetic tools to allow for the study of this organism at the molecular level. To compound this, vaccines are so effective that understanding the organism has not been required to manage disease burden [7].

Worldwide, billions of people and countless companion and production animals are protected from tetanus by immunisation with highly effective TeNT toxoid vaccines. Despite its importance, the current fermentation processes have advanced little since the 1970s. While not reported in scientific literature, industrial TeNT production is hampered by low titres and occasional batch failures (communication, Zoetis Inc.). TeNT is produced from *C. tetani* cultures grown under classical batch fermentation conditions. At the end of the fermentation, after ~150 h, the active toxin is harvested from the culture supernatant. The toxin is subsequently inactivated with formalin before it is mixed with other components for use in multivalent vaccines. The medium used for TeNT production is critical and typically consists of peptides from digested casein, soy or occasionally other plant derived peptones, which are inherently of inconsistent quality. In addition, during medium formulation,

* Corresponding author.
*E-mail address:* e.marcellin@uq.edu.au (E. Marcellin).
[1] Equal contributors.

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medium components also undergo additional poorly-controlled modifications such as calcium precipitation [8–10] and Maillard reactions [11] which exacerbate the variability in yields. Critically, it remains unclear which components of the medium are essential for toxin production and even the fundamental reason(s) for the organism to produce toxin.

Attempts to improve TeNT production using traditional rational process engineering approaches have had limited success [12]. Fed-batch processes that increase cell density and TeNT production have not been transferred to the commercial settings, while attempts to rationally design medium have resulted in reduced TeNT production [13]. To improve TeNT production, it is imperative to better understand the biology of *C. tetani* under industrial conditions; apart from the isolated example that links the tetanus toxin precursor gene, *tetX*, expression to the regulator *tetR* [14], little is known about *C. tetani* physiology and TeTN production elicitors. Typically, toxin production in clostridia is a highly regulated and complex molecular process [15]. In the case of TeNT, the gene encoding TeNT (*tetX*) must not only be expressed and translated, but the resulting proto-toxin (pTeNT) must also undergo proteolytic cleavage before the toxin becomes active [16]. Analysis of *tetX* expression and regulation, and TeNT production and maturation has been heavily reliant on comparative analysis with *Clostridium botulinum* and *Clostridium perfringens*.

To address the challenges associated with TeNT production, here we have performed a systematic study of the behaviour of *C. tetani* during bioreactor fermentations for the production of TeNT using a multi-dimensional biological analysis. Systems biology has been widely adopted for the study of model organisms such as yeast and *E. coli* [17] however, it is not widely used for the study of non-model organisms. Here we performed an in-depth time-course analysis of the fermentation that defines nutrient consumption and a 15 time-point transcriptomics profile dataset to provide a detailed 'transcriptional fermentation atlas' of the process. We have identified distinct growth phases and attribute mechanisms to explain the complex life cycle of *C. tetani* in bioreactors, which are tightly controlled through complex growth phase switches at the transcriptional and translational level. At the molecular level, three 'transcriptional stages' were identified. We believe that this research will underpin future improvements to the production of TeNT and contribute to our global understanding of the central metabolic regulation, pathogenicity, and life cycle of *C. tetani* under industrial conditions.

## 2. Results

The first step for bioprocess optimisation is the establishment of constant environmental conditions to maintain high reproducibility. To achieve high toxin yields, *C. tetani* needs to be fermented using a complex process under a tightly controlled environment. As opposed to our initial attempts in which large batch to batch variability with poor active toxin recovery was observed in flask fermentations, bioreactors provided highly reproducible results (Fig. 1A).

### 2.1. C. tetani's growth displays two exponential growth phases: exponential I and exponential II

After establishing reliable fermentations, we sampled the fermenters frequently so as to uncover a previously uncharacterised exponential growth phase followed by a second phase of lower growth rate (Fig. 1A). The fermentation begins with a period of exponential growth (Stage I), characterised by rapid production of biomass ($\mu = 0.24 \pm 0.006\ h^{-1}$), a decrease in pH and an increase in dissolved oxygen (DO) (Fig. 1C) (from 0 to 4.5%) when air is passed through the headspace. Ten hours after the start of the fermentation, cells transition to Stage II, which is characterised by a slower growth rate ($\mu = 0.083 \pm 0.005\ h^{-1}$) for the next 30 h. Growth rates were determined by dry cell weight (DCW) and optical density (OD). During this stage, pH increases to a peak of 7.2 after 25 h of the fermentation start, while DO concentrations remain stable. During Stage III (40 h–70 h) cells undergo a phase of autolysis. At this time, dramatic increases in both pH and DO are observed with pH rising from 7.2 to 8.0 and DO increase from 5% to 70%. Stage IV is the final period of toxin maturation in which the proto-toxin in the supernatant becomes biologically active. Although pTeNT must be in the supernatant following autolysis, the concentration of active toxin does not peak until 120 h, some 48 h after complete lysis is observed.

To characterise the biological process for TeNT production at the molecular level, fermenters were sampled regularly during the four growth stages (Fig. 1). Given the influence of medium composition in TeNT production yields and the lack of data supporting its design, we used a systems biology driven approach to understand the physiological program that governs the behaviour of the *C. tetani* population during fermentation. To this end, time course samples were taken for analysis of medium component consumption, namely free amino acids and vitamins (Fig. 1D and E), metal ions, and casein-derived peptides (Fig. 2A and B). Results were mapped to the fermentation profile to generate a physiological fermentation atlas. It is important to note that due to the inherent nature of the fermentation in complex medium results from the repetitions are similar but not identical. This is particularly true for the proteomics study and for the RNA-seq repeats. Variations are likely due to variability in the proteins digestion prior to MS analysis and the inherent nature of the complex fermentations (see for example ph and $pO_2$ profile for 5 replicates in Fig. 1C). For clarity and simplicity, most results will focus on the fine kinetic fermentation (the one that contains 15 time points) while results from both fermentations are presented in the figures and described in the text.

### 2.2. Growth on free amino acids

The transition from the Stage I to Stage II is associated with a depletion of a subset of free amino acids from the medium (Fig. 1D). Analysis examining nutrient consumption indicated complete or almost complete (<10% remaining) consumption of glutamic acid, aspartic acid, asparagine, threonine, serine, histidine, and tyrosine. The fastest decrease in amino acid concentration occurs between the 11 h and 13 h, particularly for histidine, glutamic acid, threonine, and serine. Aspartic acid and asparagine were consumed between 10 h and 20 h and tyrosine was consumed after 30 h. Interestingly, depletion of amino acids correlates with the transition from Stage I to Stage II. Contrarily, the concentration of Ile, Pro and Val increased along the fermentation presumably due to the activity of extracellular peptidases (Supplementary Figs. 1 and 2).

To investigate the nutritional dependence of casein-derived peptides in the fermentation, cells were fermented in a chemically defined medium containing the 20 proteinogenic amino acids plus hydroxy-L-proline, cystine [18] and glucose. The chemically defined medium supports growth at $\mu = 0.69\ h^{-1}$ despite no glucose being consumed. When using the chemically defined medium, cells stop growing after the depletion of aspartate, asparagine, glutamate, serine, histidine, methionine, leucine, lysine, arginine, threonine and glutamine while no toxin production was observed confirming the importance of casein peptides and amino acid metabolism in the *C. tetani* fermentation (Supplementary Fig. 3). Further gene expression analysis confirmed that the gene encoding for *tetX* is essentially not transcribed in CDM (data not shown).