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Insights into *Clostridium tetani*: from genome to bioreactors

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Abstract

Tetanus vaccination is of major importance for public health in most countries in the world. The World Health Organization indicated that 15,000 tetanus cases were reported in 2018 (World Health Organization, 2019). Currently, vaccine manufacturers use tetanus toxin produced by *Clostridium tetani* fermentation in complex media. The complex components, commonly derived from animal sources, introduce potential variability in cultures. To achieve replicable fermentation and to avoid toxic or allergic reactions from animal-source compounds, several studies have tried to switch from complex to chemically defined media without affecting toxin titers. The present review introduces the current knowledge on i) *C. tetani* strain diversity, whole-genome sequences and metabolic networks; ii) toxin regulation and synthesis; and iii) culture media, cultivation processes and growth requirements. We critically reviewed the reported data on metabolism in *C. tetani* and completed comparative genomic and proteomic analyses with other *Clostridia* species. We integrated genomic data based on whole-genome sequence annotation, supplemented with cofactor specificities determined by protein sequence identity, in a new map of *C. tetani* central metabolism. This is the first data review that integrates insights from omics experiments on *C. tetani*. The overview of *C. tetani* physiology described here could provide support for the design of new chemically defined media devoid of complex sources for toxin production.

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1. Introduction

Clostridium tetani is the pathogen responsible for tetanus. The disease is caused by a toxin produced by the bacterium and is not contagious. People suffering from tetanus do not develop any immunization, as the toxin level needed to induce an immune reaction is lethal. Tetanus remains a public health issue, especially in developing countries. Vaccines that are effective against tetanus are produced from inactivated *C. tetani* toxin.

C. tetani is a strictly anaerobic bacillus. Its spores are naturally present in soils, especially in warm and humid materials (ground, dejections). When these spores penetrate a wound, their germination is possible under favorable conditions (nonirrigated damaged tissues). The incubation time lasts between three and twenty-one days (World Health Organization, 2017). Bacteria stay in the necrotic wound and release the tetanus neurotoxin. This neurotoxin enters the tissues, reaches the lymphatic system and is then transported by nerves or blood to the central nervous system. The toxin blocks inhibitory neurotransmitters, causing the well-known tetanus-associated muscular rigidity and spasms (Evans and Brachmann, 1998).

The tetanus-related death rate reaches 100 % in the absence of treatment. Most of the cases are related to neonatal tetanus, occurring in newborns or their mothers when hygienic conditions are not maintained; approximately 34,000 newborns died from tetanus in 2015, mainly in low-income countries (World Health Organization, 2017). Wound-related tetanus usually occurs in elderly individuals who are not up to date on their vaccination. Thus, because of this high pathogenicity, massive preventive vaccination has to be implemented to protect the population.

The tetanus vaccine is produced from *C. tetani* culture. The produced native tetanus toxin is then inactivated with formaldehyde to produce a toxoid. Because of this inactivation, the addition of an adjuvant is required to induce an immune response without lethality (Smith et al., 2011).

For vaccine production, *C. tetani* strains are industrially grown in complex media derived from Mueller and Miller medium (World Health Organization, 1994). This medium is mainly composed of casein hydrolysate, glucose, beef heart infusion and vitamins. Since pancreatic digests of casein and beef heart infusion are undefined complex materials, they cause important batch-to-batch variability. Mueller reported halved toxin titers when using a new batch of casein digests. After analysis of samples from various stages of casein digest production, the batch-to-batch variability remained unexplained (Mueller and Miller, 1954).

Because of this variability and to avoid toxic or allergic reactions from incomplete digestion of mammalian proteins in complex media, the World Health Organization encourages all vaccine manufacturers to cultivate *C. tetani* in chemically defined media (World Health Organization, 1994).

A better understanding of *C. tetani* metabolism and physiology is required in order to simplify media components or to accomplish fermentation with high titers in chemically defined medium. This knowledge could be used to develop a chemically defined medium suitable for tetanus toxin production. This review provides a functional genomics analysis focused on relevant metabolic pathways for toxin production at an industrial scale. The regulation of toxin synthesis is not discussed in depth, as it was already reviewed by the Institute of Microbiology and Genetics (Bruggemann and Gottschalk, 2004) and the Pasteur Institute (Connan et al., 2013).

2. *Clostridium tetani* genome and central metabolism

2.1. *Clostridium tetani* strain diversity and genome sequences

To date, 43 *C. tetani* strains have been isolated, mainly from human wounds, in different geographic locations worldwide (North America, China, France) (Chapeton-Montes et al., 2019; Cohen et al., 2017; U.S. National Library of Medicine). Strains are classified into two main groups: the Harvard strains derived from the ancestral Harvard strain (Clade 1A) and the wild-type strains isolated from clinical cases (Clades 1B-H and 2). The ancestral Harvard strain was collected by the Hygienic Laboratory of the US Public Health Service by the end of the First World War. That strain was then distributed to laboratories and vaccine manufacturers from the 1920s to the 1950s. This led to the development of the Harvard family strains, which are characterized by low sporulation and high toxin production and are suitable for vaccine production. The strains E88 and CN655, isolated from the Harvard family, are commonly used in European laboratories.

The 2.8 Mb genome of the *C. tetani* reference strain was sequenced in 2003 by Brüggemann (Bruggemann et al., 2003). The genome of *C. tetani* strain Harvard E88 encodes 2,372 ORFs (28.6 % G + C content) and a unique 74-kbp plasmid (24.5 % G + C content) with 61 ORFs. That plasmid includes the gene encoding the tetanus toxin (*tent*), its transcriptional regulator *tetR*, and genes encoding virulence factors (surface layer and adhesion proteins, collagenase). On the plasmid, ABC transporter-encoding genes (CTP24-25) were found directly

downstream of a two-component system (TCS) composed of a response regulator (CTP21) and a histidine kinase (CTP22). Brüggemann et al. (2004) suggested that this two-component system, located 25 kb before the tetanus toxin gene, might regulate the transcription of these ABC transporter genes. Virulence factors were also found on the chromosome, such as tetanolysin O (CTC1888), hemolysin (CTC586, CTC1574) and fibronectin-binding protein (CTC164, CTC471, CTC1606). These proteins help *C. tetani* infect damaged tissues.

Among the 20 genes annotated as surface layer proteins in the E88 genome, a surface layer protein encoded by the chromosomal *slpA* gene (CTC462) was characterized by Qazi et al. (2007). The results showed a variability in its molecular weight depending on the strain (160 kDa in CN655, 180 kDa in the clinical isolate NC06336-07, 160 to 180 kDa in the three clinical isolates CTHCM 19, 22 and 25) compared to a 118 kDa predicted protein. No glycosylation was found to explain this variability (Qazi et al., 2007). Such observations were already described by Takumi et al. (1991) with the characterization of the protein from the AO174 strain with a molecular weight of 140 kDa. Takumi et al. (1991) found that SlpA had an unusually low proline content and showed various isoelectric forms from pH 4.0 to 4.5. This surface layer protein presented an antigen, making immunological detection possible (Takumi et al., 1991).

Brüggemann et al. (2004) identified a heme oxygenase (CTC2478) in *C. tetani* that could confer oxygen tolerance to bacteria during wound infection by creating a local anaerobic environment. This enzyme was also found in another *Clostridium* wound colonizer (*Clostridium perfringens*) but not in the other pathogenic or nonpathogenic *Clostridia*. Thus, *C. tetani* is an anaerobic microorganism but is reported as an aerotolerant species, similar to *C. perfringens* (Bruggemann et al., 2004). Dedic et al. (1956) succeeded in growing *C. tetani* under aerobic conditions by the addition of cobalt (as $Co(NO_3)_2$). Under anaerobic conditions, cobalt supplementation did not influence growth. The authors suggested that cobalt addition reduced the oxygen partial pressure of the cultivation medium (Dedic and Koch, 1956); however, this observation could also be related to the presence of heme oxygenase.

A particularly high number of sodium ion-dependent genes (35) were found in the *C. tetani* chromosome, and these genes might be related to the wound colonization capacity of this organism. A gene cluster (CTC1337-1352) homologous to the Mrp system was found on the chromosome. This gene cluster, absent in other *Clostridia,* provides resistance to high levels of sodium and potassium ions and regulates the intracellular pH. The sodium-motive force can also be driven by a Rnf-like complex (RnfC, RnfD, RnfG, RnfE, RnfA and RnfB encoded

by CTC1019-1024) that transports the sodium ions out and regenerates NADH and oxidized ferredoxin. This sodium flow could then potentially be used to conserve energy through a V-type sodium ATP synthase (CTC2326-2332), producing ATP (Bruggemann and Gottschalk, 2004). E88 also harbors the gene encoding the CodY protein (CTC1260), which is a global regulator. This protein is known to indirectly downregulate toxin production in *Clostridium difficile* (Dineen et al., 2007) but positively regulate toxin synthesis in *C. perfringens* (Li et al., 2013) and *Clostridium botulinum* (Zhang et al., 2014).

There are 28 transposase genes in the E88 genome, but most of them seem to be nonfunctional because of damaged ORFs. Only a few regions have a high G + C content (approximately 50 %): six rRNA gene clusters and ribosomal protein-encoding genes (Bruggemann et al., 2003).

To date, the whole-genome sequences of the 43 isolated strains have been reported (U.S. National Library of Medicine). All the genome sequences were compared to the genome of the model strain E88. Analysis showed that genetic identity among variants was high. The toxin-encoding genes have similar sequences with 99.3–99.4 % identity among all Harvard strains harboring the tetanus plasmid (two strains lost their plasmid and thus their pathogenicity). These Harvard and wild-type strains also harbor an identical toxin transcriptional regulator gene sequence with 100 % identity (Cohen et al., 2017). Strain-specific proteins are encoded in their prophage regions. Other differences among these strains are found in their CRISPR/Cas loci and spacer regions. They mainly encode a set of fitness functions that protect these strains from environmental stress or new prophage infections (Bruggemann et al., 2015). Among the 43 *C. tetani* genome sequences reported, Chapeton-Montes et al. (2019) analyzed 38 genome sequences and showed that the core genome represents 77 % of the E88 genome. The 38 strains share 1,266 coding sequences (CDS; 32 % of the total CDS). The Harvard strains only differ by 292 single-nucleotide polymorphisms (SNPs) in their whole genomes and share a 100 % identical tetanus toxin at the protein level. They harbor strain-specific genes, encoding “a plasmid-like element carrying a toxin-antitoxin system, a gene cluster encoding surface-layer proteins, an iron transport system and a putative cell wall/spore coat/envelope/membrane modification system” (Chapeton-Montes et al., 2019). Phylogenetic analysis showed that the *C. tetani* plasmid evolved together with the chromosome and is not a recent genomic element. This reflects the genomic stability of *C. tetani* (Chapeton-Montes et al., 2019; Cohen et al., 2017). The *C. tetani* genome is considered to be stable in comparison with the genomes of other species, such as *C. botulinum*

(Chapeton-Montes et al., 2019). The strains used for *C. tetani* experiments in the laboratory belong to the same genomic subgroup (the Harvard family, clade 1A) and have identical toxin- and toxin transcriptional regulator-encoding genes.

2.2. *Clostridium tetani* central metabolism

The substrates most commonly used by *C. tetani* are amino acids, as many genes for their transport and degradation were identified in its genome (Table 1-A). The amino acid transport seems to be mainly sodium dependent, occurring through symporters (Bruggemann and Gottschalk, 2004). *C. tetani* transports and catabolizes numerous amino acids. The regulation of this gene expression was, up to now, poorly understood. Recently, Orellana et al. (2020) used a time-course comparative transcriptomics approach to better understand how the amino acid degradation pathways were expressed when the E88 strain was grown in complex medium with or without supplementation with five amino acids. The results showed that histidine and aspartate were mainly degraded to glutamate, which was degraded to acetate, butyrate, pyruvate and ammonium via the methylaspartate pathway. Serine was degraded mainly to pyruvate via the serine ammonia-lyase pathway (CTC1981-1982), threonine to glycine and acetaldehyde, and tyrosine to pyruvate and phenol via the tyrosine phenol lyase pathway (CTC818). Methionine was found to be mainly converted to 2-oxobutanoate and propionyl-CoA. However, other degradation pathways of these amino acids were found to be induced at the transcriptional level but to a lesser extent, and this induction was dependent on the cultivation phase (Table 1-B)

As *C. tetani* assimilates many amino acids, its genome lacks biosynthetic pathways for at least phenylalanine, histidine, isoleucine, lysine, leucine, methionine, tryptophan and valine, causing amino acid auxotrophy. This is typical for pathogenic bacteria with small genomes: they do not develop pathways for amino acid biosynthesis because they live in a host (Yu et al., 2009). In addition to amino acid transport and degradation genes, *C. tetani* harbors numerous extracellular and intracellular peptidase- and (phospho)lipase-encoding genes. This set of peptidases, which are characteristic of pathogenic *Clostridia*, provides free amino acids to the bacteria. Genome sequence analysis data indicates that *C. tetani*, similar to *Clostridium butyricum,* could ferment glycerol to produce 1,3-propanediol (Gonzalez-Pajuelo et al., 2006) (glycerol kinases CTC1758, CTC2462; glycerol-3-phosphate dehydrogenases CTC596, CTC1139, CTC1808, CTC2436; glycerol dehydratases CTC936, CTC1449), ethanolamine

187 (gene cluster CTC2163-2181) and inositol (gene cluster CTC508-514) (Bruggemann and
188 Gottschalk, 2004).

189 **Table 1-A: Annotation of the amino acid transporter and degradation genes**

Amino acid	Amino acid transporter gene annotation	Amino acid degradation gene annotation
Alanine	CTC564, CTC1172, CTC1975	CTC695
Arginine	Not annotated	CTC1763
Aspartic acid	Not annotated	CTC561-562, CTC824-825, CTC1294, CTC1309, CTC1876, CTC2383-2384
Cystine / Cysteine	CTC559	CTC1050
Glutamic acid	CTC822, CTC2306, CTC2324	CTC1295, CTC2563, CTC2565, CTC2567-2568
Glutamine	CTC559	CTC171
Glycine	CTC564, CTC1172, CTC1975	Not annotated
Histidine	Not annotated	CTC2318, CTC2321
Leucine	CTC787, CTC1868, CTC2088	CTC1738
Lysine	Not annotated	CTC890
Methionine	CTC1355	CTC2530
Proline	CTC1190	Not annotated
Serine	CTC1514, CTC2307	CTC1981-1982
Threonine	CTC1514, CTC2307	CTC2624
Tryptophan	CTC1190	CTC1509
Tyrosine	CTC819	CTC818

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Table 1-B: End products of the major and minor degradation pathways

Amino acid	Major degradation pathways	Minor degradation pathways
Aspartate	Glutamate + Oxaloacetate	Ammonium + Fumarate
Glutamate	Acetate + Butyrate + Pyruvate + Ammonium	2-oxoglutarate + Ammonium n-Formimino-L-Glutamate
Histidine	Glutamate	Histamine
Methionine	2-Oxobutanoate + Propionyl-CoA Propionate + ATP + Ammonium	S-Adenosyl-L-Methionine
Serine	Pyruvate + Ammonium	L-Cysteine
Threonine	Glycine + Acetaldehyde	Propionyl-CoA Propionate + ATP + Ammonium
Tyrosine	Pyruvate + Phenol + Ammonia	-

C. tetani ferments only glucose as a sugar source. The Harvard strains did not take up galactose, fructose, mannose, maltose, sucrose and lactose in complex (casein, yeast extract) medium (Martinez and Rittenberg, 1959). *C. tetani* harbors the genes encoding enzymes for glycolysis (CTC341, CTC378-382, CTC507, CTC2404, CTC2489-2490, CTC2515, CTC2637), glucose PTS transport (CTC278, CTC1771), or sodium symporter activity (CTC1237) but none for polysaccharide degradation. Unlike other *Clostridium* genomes, the *C. tetani* genome also contains genes from the pentose phosphate pathway (CTC228, CTC307, CTC1227, CTC1332, CTC1864-1865 and many putative genes encoding hydrolases). The degradation of glucose and many amino acids results mainly in pyruvate formation. No genes encoding citrate cycle enzymes were found in the *C. tetani* genome.

Pyruvate is converted to acetyl-CoA through pyruvate ferredoxin oxidoreductase. This enzyme reduces ferredoxin, which is then reoxidized by a hydrogenase, releasing hydrogen. Acetyl-CoA can then be converted to acetate or butyrate through complete acetate and butyrate biosynthetic pathways. The *C. tetani* genome harbors one 3-hydroxybutyryl-CoA dehydrogenase (Hbd), two crotonases (Crt) and five homologous butyryl-CoA dehydrogenases (Bcd). Acetyl-CoA catabolism can also result in ethanol and butanol production (Bruggemann and Gottschalk, 2004). It was experimentally demonstrated in *Clostridium acetobutylicum* (Yoo et al., 2015) and *Clostridium kluyveri* (F. Li et al., 2008) that butyryl-CoA dehydrogenase (Bcd) is an NADH- and ferredoxin-dependent enzyme. The Bcd enzyme in *C. tetani* (CTC2426) showed 82.6 % and 74.9 % identity with the Bcd enzyme

from *C. acetobutylicum* and *C. kluyveri,* respectively, strongly suggesting its NADH and ferredoxin dependence in *C. tetani*. The Hbd enzyme was shown to be NADH dependent in *C. acetobutylicum* and NADPH dependent in *C. kluyveri* (Yoo et al., 2020). The Hbd CTC2423 from *C. tetani* has a higher sequence identity with *C. acetobutylicum* (80.5 %) than with *C. kluyveri* (68.9 %), suggesting that it is an NADH-dependent enzyme. Both the AdhE1 and AdhE2 aldehyde-alcohol dehydrogenases from *C. acetobutylicum* were biochemically characterized *in vitro* and shown to be strictly NADH dependent (Yoo et al., 2015). Both enzymes have dual functionality, participating in the conversion of i) butyryl-CoA to butyraldehyde and ii) acetyl-CoA to acetaldehyde. A similar enzyme, CTC1366, sharing 68.2 % amino acid identity with AdhE1 and 67.2 % amino acid identity with AdhE2, was identified in *C. tetani.* The three enzymes showed a conserved GCGXWG domain, which is commonly involved in coenzyme binding (Fontaine et al. 2002). Thus, on the basis of amino acid identity, aldehyde-alcohol dehydrogenase in *C. tetani* is likely NADH dependent. Finally, a butanol dehydrogenase, CTC408, catalyzing the conversion of butyraldehyde to butanol, was also identified in *C. tetani*. This enzyme shares 66.8 % amino acid identity with BdhA and 63.8 % amino acid identity with BdhB from *C. acetobutylicum*, which were biochemically demonstrated *in vitro* to be NADPH dependent (Yoo et al. 2015). Thus, butanol dehydrogenase in *C. tetani* could be considered to be NADPH dependent. Extrapolation to *C. tetani* based on the above % amino acid identity suggests that butanol production could be dependent on NADH and NADPH in *C. tetani* as well.

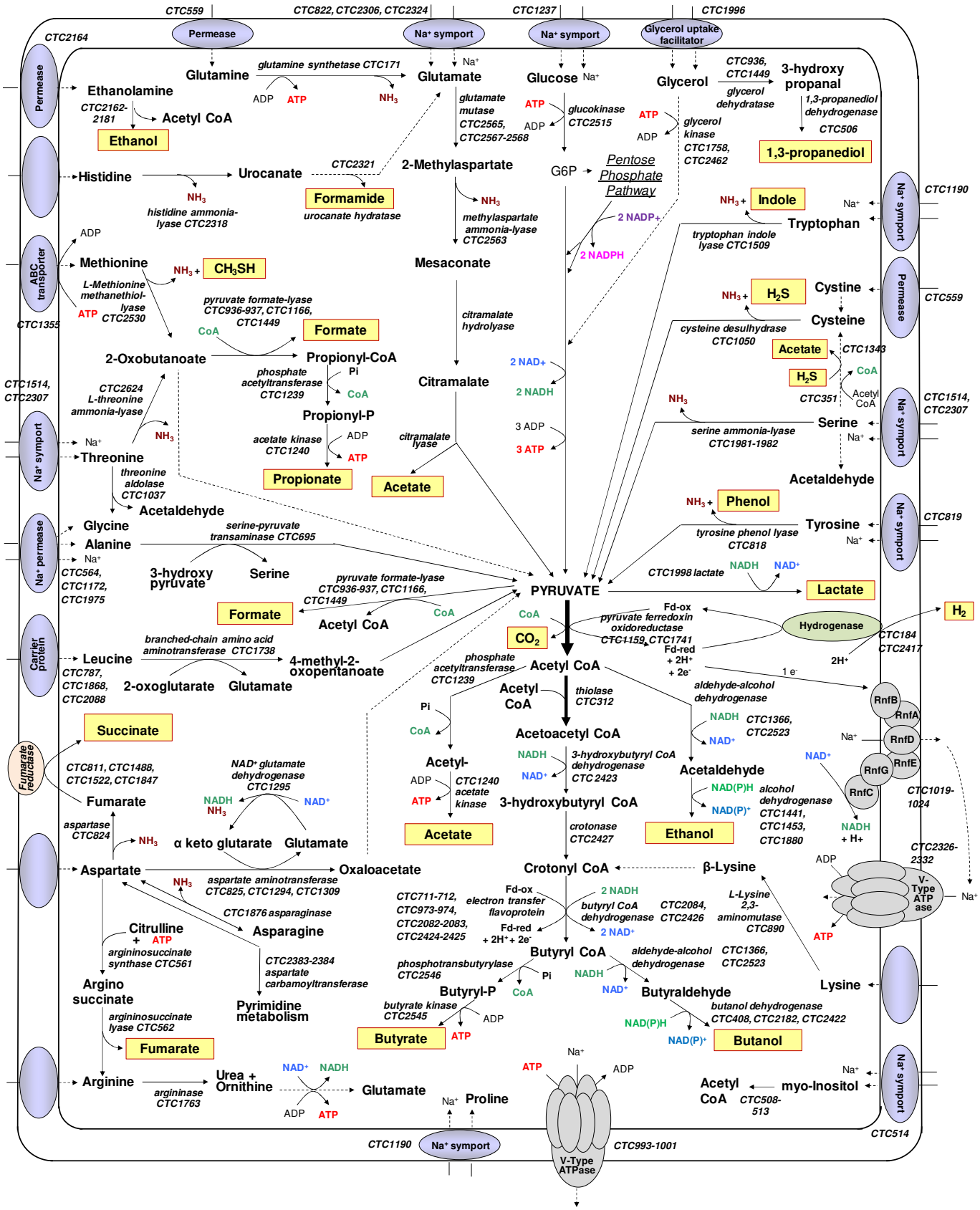

Figure 1: *Clostridium tetani* central metabolism

Yellow boxed compounds are fermentation products that are released in the medium

The general metabolic network in *C. tetani* is outlined in Figure 1. The enzymes involved in this metabolism are detailed with the number for the encoding gene. Their *in vivo* functionality has not been demonstrated. This metabolic map is not exhaustive. However, some uncertainties remain. Several amino acid transporters have not been identified (histidine, aspartate, arginine and lysine transporters). The glutamate/glutamine degradation pathway that functions through methylaspartate was first shown to be functional in *C. tetani* NCTC 5404 using C-labeled glutamate (Buckel and Barker, 1974). In 2016, Licona-Cassani et al., using in-depth transcriptional analysis, observed high expression of the methyl aspartate mutase cluster genes during the exponential phase, suggesting that glutamate is probably also metabolized via the mesaconate pathway in the E88 strain.

2.3. *Clostridium tetani* bioenergetics

Twenty-one iron-sulfur proteins with a [4Fe4S] core pattern and twenty-six homologous flavoproteins were found in *C. tetani*, suggesting a significant role of these electron transport proteins in metabolism. Among these iron-sulfur proteins, ferredoxins take part in electron transfer in *C. tetani*. Ferredoxin is reduced during the conversion of pyruvate to acetyl-CoA. Reoxidation of reduced ferredoxin generates an H^+ flow, which can be converted to dihydrogen by hydrogenases or used by ferredoxin NAD^+ oxidoreductases such as the Rnf complex. In addition, FeS clusters are found, in particular, in a membrane-bound system (CTC1019-1024) homologous to the Rnf complex (*Rhodobacter*-specific nitrogen fixation), described in acetogenic bacteria as a respiratory enzyme complex catalyzing the oxidation of reduced ferredoxin and the reduction of NAD^+, generating a transmembrane ion gradient. In *Acetobacterium woodii*, the Rnf complex was proposed to be coupled to the translocation of sodium ions across the cytoplasmic membrane (Schuchmann and Muller, 2014). Thus, the Rnf-like system in *C. tetani* is suspected to maintain the sodium-motive force in the cell, which is essential for amino acid transport and for transport of electron flow from ferredoxin activity to regenerate the NADH pool. The (V)-type ATPases are the main ATPases in *C. tetani* (CTC993-1001, CTC2326-2332). The ATPase CTC993-1001 also participates in the transport of Na^+ ions out of the cell, reinforcing the sodium-motive force of the Rnf-like system. This sodium transport is then combined with energy dissipation. No F_0-F_1-type ATPase was found in *C. tetani*, which is unusual. Nonetheless, in contrast to other *Clostridia*, *C. tetani* harbors a gene cluster (CTC2326-2332) encoding a V-type sodium ATP synthase homologous to the archaeal-type ATP synthase (Bruggemann and Gottschalk, 2004). This

complex could use the extracted sodium from the Rnf system to conserve energy with ATP synthesis. Licona-Cassani et al. (2016) showed, by generating a transcriptional molecular map of an E88 culture on complex medium, the activation of genes encoding Rnf complex proteins, a flagellum-specific ATP synthase, calcium-transporter ATPases and genes associated with V-type ATPase synthase.

An H^+ pump (V-Type pyrophosphatase CTC383), coupled with the cleavage of inorganic pyrophosphate, was also found in *C. tetani*, which is uncommon in bacteria. In addition, a specific sodium ABC transporter (CTC1485) and several H^+/Na^+ antiporters (CTC567, CTC901, CTC1183, CTC1423, CTC1853, CTC2161, CTC2520, CTC2529) were identified in the genome. Brüggemann et al. (2004) suggested that this predominant sodium ion bioenergetic profile could be linked to main fermentation pathways in *C. tetani*: the amino acid utilization pathways.

3. Tetanus toxin regulation and synthesis

3.1. Tetanus toxin regulation

3.1.1 Toxin regulation by an alternative sigma factor

The genes encoding the tetanus toxin and its regulator are located on the mega-plasmid of *C. tetani* (Raffestin et al., 2005): i) the *tetX* gene (also written *tent*) encodes the toxin, and ii) the *tetR* gene, which is located immediately upstream of *tetX*, encodes the regulator of the tetanus toxin. TetR is an alternative sigma factor (from group 5 of the σ^{70} family) that positively regulates the expression of *tetX*. It binds to an enzyme, the RNA polymerase core enzyme, to initiate the transcription of the *tetX* gene. The core enzyme and its sigma factor, combined with the *tetX* promoter, are necessary for tetanus toxin production. The tetanus toxin locus is described in Figure 2 (Raffestin et al., 2005).

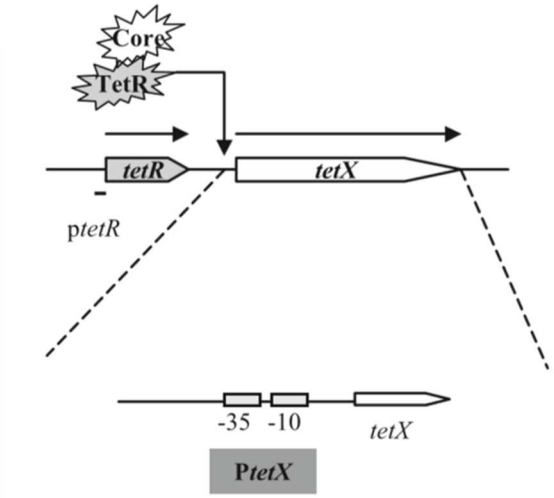

Figure 2: Genetic organization of the tetanus toxin locus in *Clostridium tetani* from Raffestin *et al.* (Raffestin et al., 2005)

The promoter of the tetanus toxin gene (PtetX), transcribed by TetR, is expanded with conserved sequences -35/-10 (gray boxes). The solid bar represents a putative promoter that is not transcribed by TetR, located upstream of the tetR gene (ptetR) (Raffestin et al., 2005).

The genetic organization of the tetanus toxin operon is similar to that of the botulinum toxin operon. Indeed, the botulinum sigma factor BotR/A showed 60 % amino acid identity with TetR. Moreover, *in vivo* overexpression of the exogenous BotR/A in *C. tetani* cells positively regulated TetX synthesis, illustrating the functional similarity between TetR and BotR/A (Marvaud et al., 1998).

3.1.2. Toxin regulation by Two-Component Systems

Tetanus toxin synthesis is regulated by a complex network of TCSs. For toxin synthesis, at least two positive regulator TCSs and one negative regulator TCS were identified by Chapeton-Montes et al. (2020). The first positive regulator TCS is located 25 kb upstream *of tetX* on the plasmid, close to ATP-binding protein-encoding genes, but does not show any homology with other bacteria. The other positive regulator TCS is located on the chromosome and is putatively related to autolysis. These two TCSs are suspected to indirectly regulate toxin synthesis since their proteins do not bind to TetR or TetX promoters. In contrast, the negative regulator TCS directly represses toxin synthesis by binding to the TetR and TetX promoters. On the basis of homology, this TCS, located on the chromosome, is involved in cell division. Some other TCSs were suspected to act indirectly on toxin concentration by altering the cell membrane or controlling toxin secretion (Chapeton-Montes et al., 2020).

3.1.3 Toxin regulation related to virulence factors and cell division

Chapeton-Montes et al. (2020) showed that toxin synthesis was also regulated by CodY. This protein, often involved in toxin and virulence regulation in other Gram-positive pathogenic bacteria, exhibited binding to the *tetX* promoter. This increased *tetX* transcription, which positively regulated toxin synthesis. As CodY is known to respond to starvation conditions, the authors suggested that this toxin regulation could be related to nutritional stress (Chapeton-Montes et al., 2020). Comparative transcriptional analysis of E88 grown in complex medium supplemented or not with an amino acid mixture suggested also that toxin production and virulence factors were related (Orellana et al., 2020). Specifically, the decrease in expression of virulence-related surface/cell-adhesion genes (CTC769-770, CTC772, CTC776-777) and flagellar genes considered as virulence-related genes in other pathogens (CTC1653-1679) affected toxin production. However, the genes encoding the homologous TCS of the virulence genes VirS/VirR in *C. perfringens* showed no difference in expression, regardless of the tetanus toxin production level.

C. tetani phenotypes seemed to depend on toxin synthesis, with the long-chained filaments appearing in the culture with the highest toxin production (Orellana et al., 2020). Yet, the expression of cell division and cell wall elongation genes was not linked to toxin synthesis when the E88 strain was grown in complex medium supplemented or not with a mixture of five amino acids. However, the transcription of the genes (CTC280, CTC316, CTC595, CTC2066) encoding autolysin proteins, which are involved in cell separation and cell shape, was inhibited under high toxin production, explaining these different cell morphologies.

3.2. Toxin production and maturation

The tetanus toxin is synthesized in the bacterial cytosol as a 151-kDa chain protein (1315 amino acids). Its maturation process is illustrated in Figure 3.

After translation of the RNA to protein, the first methionine residue is removed, and two cysteine disulfide bridges are formed. The tetanus toxin is released in the culture medium. The toxin peptide chain is then hydrolyzed by a 27-kDa protease in the culture broth, resulting in a toxin composed of a 52-kDa light chain and a 98-kDa heavy chain (T. B. Helting et al., 1979). One disulfide bond links the heavy chain to the light chain, and another one forms a loop on the heavy chain. The single-chain toxin is cleaved between Glu449 and Asn450. However, toxins were also found in the culture broth as cleaved from Glu449 to Ser457,

missing the junction residue. This protein nicking considerably increases its toxicity (Krieglstein et al., 1991).

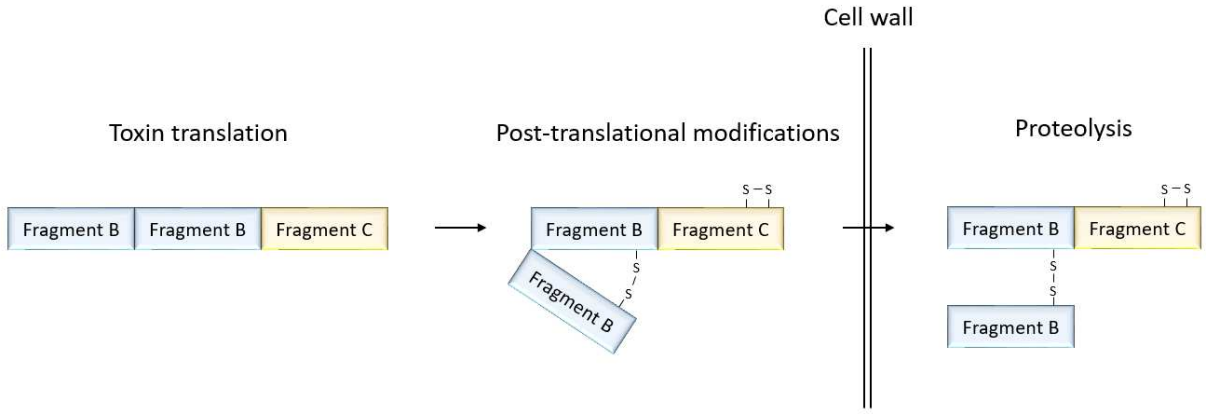

Figure 3: Maturation process of *Clostridium tetani* toxin

Helting et al. (1979) (T. B. Helting et al., 1979) identified three active proteases in the culture broth. The one that cleaved the single-chain toxin had a molecular weight of 27 kDa. It was the main active component *in vitro*: 3 ng of this enzyme can cleave 50 µg of intracellular toxin. The activity of the second protease was not identified. The last enzyme was one that hydrolyzes glycyl-histidine without modifying the toxin conformation. The protease cleaving the single-chain toxin had optimal *in vitro* enzymatic activity in the pH range 6-7. The two other enzymes worked better from pH 7 to pH 9.

In 1977, Helting et al. defined tetanus toxin as a two-fragment protein: fragment B (95-kDa, both on light and heavy chains) and fragment C (47-kDa, on heavy chain). Fragment C is the toxin part that triggers the strongest antigenic reaction, although it is not toxic (Helting et al. 1977).

Schiavo et al. (1992) identified one zinc atom linked to the light chain playing a catalytic role. This atom is linked to a two-histidine motif, characteristic of metalloendopeptidases. Without zinc, the tetanus toxin cannot inhibit neurotransmitters and therefore cannot cause disease (Schiavo et al., 1992).

The tetanus toxin is very stable among the different strains because of its preserved amino acid sequence. Only a few changes in its amino acid composition were identified in clinical isolate strains. Its histidine pattern (HExxH) and its binding residues are always conserved (Chapeton-Montes et al., 2019).

3.3. Toxin tridimensional structure

The crystal structure of the full-length tetanus toxin was obtained for the first time by Masuyer et al. (2017) using X-ray crystallography (the subunit crystal structures were previously described separately). The tridimensional structure of the tetanus toxin is presented in Figure 4. As previously described, the light chain (on fragment B) is the catalytic domain. The translocation domain (which is also part of fragment B) plays a role in transporting the toxin toward neurotransmitters. The binding domain (which is part of fragment C) binds to the neurons. Therefore, antibodies directed against fragment C are the most potent: they block the binding of the toxin to neurons (Masuyer et al., 2017).

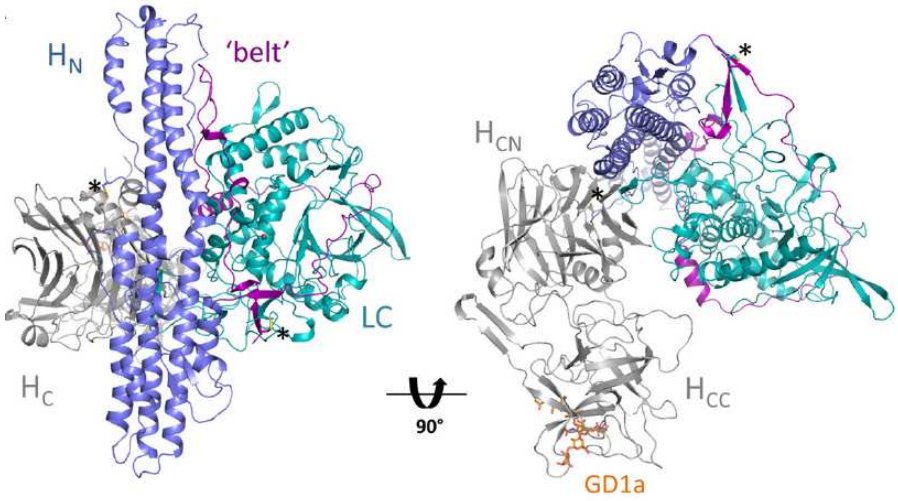

Figure 4: Overall structure of tetanus toxin from Masuyer *et al.* (Masuyer et al., 2017)

Green: light chain (LC). Blue: translocation domain (Licona-Cassani et al.). Gray: binding domain (H_C). Pink: belt surrounding the light chain. Asterisk: disulfide bridges. Orange: GD1a ganglioside (receptor of tetanus toxin over the neurons)

With solution scattering analysis, Masuyer et al. (2017) showed that the tetanus toxin structure is pH dependent. At acidic pH (under 5.5), the toxin is in a compact form (Figure 4). Its structure adopts an extended conformation at pH values greater than 6.3. Between these two values, the toxin is in a semiopen conformation (Masuyer et al., 2017).

4. Analytical methods for toxin detection

4.1. Flocculation method

Historically, the amount of tetanus toxin has been visually evaluated using the Ramon flocculation method (Ramon, 1923). This method was first designed for diphtheria toxin titration and was subsequently adopted for tetanus toxin. This titration consists of an antigen-antibody reaction. The antigen is located on the toxin, and the antibody is an antitetanus serum. This method requires a range of standard test tubes prepared with a serum in which the concentration of antitetanus antibody is well known. Then, after sample addition, white flocs appear in the tube with the best balance between toxin and serum. It is the first test tube in which flocculation occurs that indicates the toxin titer of the sample. For faster flocculation, tubes should be incubated in a water bath at up to 45°C. Titers determined with the Ramon method are expressed in Lf/mL of supernatant (where Lf stands for limit of flocculation). As it was for decades the only titration method for tetanus toxin, it is the only method currently approved by health authorities.

4.2. Minimal lethal dose

Few researchers use the minimal lethal dose value (MLD/mL of supernatant) to evaluate the toxin content and activity. This test, often performed on mice, consists of injecting diluted supernatant into animals. Then, knowing the greatest dilution associated with animal death, it is possible to calculate the MLD units contained in one sample. For reference, the MLD for humans is 2.5 ng/kg (Gill, 1982). The MLD method yielded more accurate results. However, as it requires animal resources and is labor intensive, this method is not often used.

4.3. Enzyme-linked immunosorbent assay

A routine test for tetanus antibody detection has been described by the World Health Organization (World Health Organization, 2013). This test, based on a capture enzyme-linked immunosorbent assay (ELISA), determines the tetanus toxoid content in the vaccine. The procedure consists of coating a monoclonal antibody on microplates. This antibody links the heavy chain of the toxoid. Then, wells are filled with standards and samples. Then, a polyclonal antibody is dropped in the wells. This antibody is recognized by a labeled secondary antibody, which is visualized by a colorimetric substrate. The optical density of the

sample is correlated in log scale with the amount of toxoid in the sample. The detection limit of this method is 0.002 Lf/mL.

As the toxin and toxoid have the same antibody recognition sites, this test can be performed for toxin quantification. Monoclonal and polyclonal antibodies can be switched using the appropriate secondary antibody. This method is more accurate for toxin determination than the flocculation test or MLD measurement. However, it is not routinely used because it is not approved by the World Health Organization.

5. *Clostridium tetani* fermentation processes

5.1. Culture media for *Clostridium tetani*

A *C. tetani* growth medium was first designed in 1942 in the Mueller and Miller laboratory using a Harvard strain. Until the 1960s, their work focused on improving the final toxin titer of *C. tetani* cultures in this medium. The final Mueller and Miller medium was mainly composed of casein hydrolysate (usually NZ-Case), glucose and vitamins. Table 2 details the composition differences between the final Mueller and Miller medium and the modified medium made by Latham *et al.* in 1962 (Latham et al., 1962). Latham's medium was also called Massachusetts medium.

Table 2: Comparison of the compositions of Mueller and Miller, Massachusetts, soy and TGY media

Constituents per liter	Final Mueller and Miller medium (Mueller and Miller, 1954)	Massachusetts medium (Latham et al., 1962)	Soy medium (Demain et al., 2005)	TGY medium (Chapeton-Montes et al., 2020)
Digest of casein	22.5 g	25 g	-	30 g
Beef heart infusion	50 mL	-	-	-
Yeast extract	-	-	-	20 g
Soy peptone	-	-	34 g	-
Glucose	11 g	8.0 g	7.5 g	5 g
NaCl	2.5 g	2.5 g	5.0 g	-
Na_2HPO_4	2 g	-	0.5 g	-
KH_2PO_4	0.15 g	-	0.175 g	-
$MgSO_4$	0.15 g	0.1 g	0.024 g	-
Cystine	0.25 g	0.125 g	-	-
Cysteine	-	-	0.125 g	-
Cysteine HCl				0.5
Tyrosine	0.5 g	-	0.125 g	-
Calcium pantothenate	1.0 mg	1.0 mg	-	-
Uracil	2.5 mg	1.25 mg	-	-
Nicotinic acid	-	0.25 mg	-	-
Thiamine	0.25 mg	0.25 mg	-	-
Riboflavin	0.25 mg	0.25 mg	-	-
Pyridoxine	0.25 mg	0.25 mg	-	-
Biotin	2.5 µg	2.5 µg	-	-
Vitamin B_{12}	-	0.05 µg	-	-
Reduced iron (powder)	0.5 g	-	0.5 g	-
$FeCl_3$. 6 H_2O	-	32 mg	-	-
pH adjustment (before autoclave)	7.0 - 7.2	7.0 ± 0.2	6.8	7.5

More than thirty years later, Demain et al. developed a plant medium free of animal-source compounds without milk hydrolysate. The raw materials were replaced by Quest Hy-Soy®

peptone (Table 2) (A. L. Demain et al., 2005). However, due to its high toxin production performance (70 to 120 Lf/mL) and because it does not contain beef heart infusion, Massachusetts medium remains the most commonly used medium for *C. tetani* cultures in laboratories and for industrial toxin production (Table 3).

455 **Table 3: Performance comparison in different culture modes**

Source	Strain	Cultivation mode and volume	Cultivation medium	Gas stripping	Growth	Toxin
(Zacharias and Bjorklund, 1968)	Strain 107 (Harvard strain derivative)	Bioreactor 1 L Continuous mode	Modified Massachusetts medium	N_2 gassing	0.125 h^{-1} dilution rate	120 Lf/mL ± 10 Lf/mL
(Jagicza et al., 1981)	Harvard strain	Bioreactor 400 L Batch mode	Mueller and Miller medium	N_2 surface flushing for 16 h, then air flow	$X_{max} = 10^8$ cells/mL	55 Lf/mL
(Gutiérrez et al., 2005)	Massachusetts strain (Harvard strain derivative)	Bioreactor 5 L Batch mode	Modified Massachusetts medium	N_2 bubbling during expo. growth, then surface aeration	$\mu = 0.46$ h^{-1} X_{max} = 0.78 g/L	73 Lf/mL
(Demain et al., 2007)	Wyeth-Lederle Vaccine and Pediatrics strain	Bioreactor 0.8 L Batch mode	Modified soy medium	N_2 bubbling	Not reported	48 Lf/ml
(Fratelli et al., 2010)	Harvard-Caracas (Harvard strain derivative)	Bottles 4 L Fed-batch mode	Modified Massachusetts medium	None	X_{max} = 4.34 g/L	70 Lf/mL
(Muniandi et al., 2013)	Harvard strain derivative	Bioreactor 400 L Batch mode	Modified Mueller medium	Air surface flow	OD_{max} = 2.3	70 Lf/mL
(Licona-Cassani et al., 2016)	E88	Bioreactors 1 L and 5 L Batch mode	Modified Massachusetts medium	N_2 surface flushing for 4 h, then air flow	Expo: μ = 0.24 h^{-1} Slow: μ = 0.08 h^{-1}	30.2 Lf/mL ± 2.9 Lf/mL
(Chung et al., 2016)	Harvard strain derivative	Bioreactor 50 L Batch mode	Modified Massachusetts medium	Punctual N_2 surface flow, then air flow	X_{max} = 3 g/L	80 Lf/ml
(Chawla et al., 2016)	Harvard strain derivative	Bioreactor 1 L Batch mode	Modified Massachusetts medium with soy	Not reported	Not reported	97.9 ± 3.3 Lf/mL

Abbreviation: Lf/mL, limit of flocculation per milliliter (tetanus toxin unit); μ, specific growth rate; X_{max}, maximum biomass concentration; OD_{max}, maximum optical density

5.2. *Clostridium tetani* growth phases in batch mode

C. tetani is mainly cultivated under batch mode in closed tubes or bottles. Only the end biomass and final toxin titer were reported, with no information on growth or toxin kinetics. Some bioreactor cultures were also described (Table 3). Jagicza et al. (1981) reported an industrial culture of the Harvard strain in Mueller and Miller medium. The biomass grew for three days from 10^5 to 10^8 cells/mL, with a pH decrease from 7.3 to 6.6. The toxin was intracellularly produced after one day of culture until the end of growth. The toxin was then released in the medium at a final toxin titer of 55 Lf/mL. A similar growth profile was also observed in a closed flask by Fratelli et al. (2005) with a maximal biomass concentration of 4.4 g/L and a final toxin titer of 40 Lf/mL using the Harvard-Caracas strain grown in Massachusetts medium (Fratelli et al., 2005). In 2016, Licona-Cassani et al. reported batch cultivation of the E88 strain on Massachusetts medium supplemented with cystine, uracil, vitamins and traces amounts of $FeCl_3$ (Table 3). During cultivation (in five independent experiments), regular sampling was applied to rigorously follow the growth of *C. tetani* in the bioreactor. Two distinct growth phases were observed: i) an exponential growth ($\mu = 0.24\ h^{-1}$) phase leading to a decrease in pH (from 6.8 to 6.6), lasting 10 h and exhibiting preferential consumption of free amino acids and ii) a slower growth ($\mu = 0.083\ h^{-1}$) phase with an increase in pH (from 6.6 to 7.0), lasting 30 h, and associated with the consumption of peptides and glucose. The main virulence factor genes were activated, including *tetX*. The growth was then followed by a cell autolysis phase, lasting 30 h, with a decrease in optical density and an increase in pH (from 7.0 to 8.0). This autolysis appeared to be related to the physiological cell state. These five cultivations resulted in the production of 30.2 ± 2.86 Lf/mL toxin (Licona-Cassani et al., 2016), which is lower than the values for all other batch cultures reported in the literature (Table 3).

5.3. Continuous culture of *Clostridium tetani*

A continuous culture of a *C. tetani* Harvard derivative strain (strain 107) was reported in the literature. In 1968, Zacharias and Björklund studied the effects of different i) dilution rates ($0.030\ h^{-1}$ to $0.169\ h^{-1}$), ii) temperatures (32°C to 39°C), iii) pH values (5.9 to 9.5) and iv) salts (0.1 g/L KCl, 0.15 g/L $CaCl_2$) in a modified Massachusetts medium (all concentrations

reduced by half). For each culture, one of these parameters was varied. The best toxin titer (120 Lf/mL toxin) was reached at a dilution rate of 0.125 h^{-1}, a temperature of 34°C and a pH of 7.4 in the modified Massachusetts medium supplemented with 0.1 g/L of potassium chloride. Growth rates from 0.03 h^{-1} to 0.125 h^{-1} resulted in similar biomass and toxin production, with a biomass concentration of 0.7 g/L and a toxin titer of 70-75 Lf/mL. Potassium was used to increase membrane permeability and thus toxin release in the extracellular cultivation medium. The element controlling the growth at steady state was not described (Zacharias and Bjorklund, 1968).

5.4. Fed-batch culture of *Clostridium tetani*

In 2010, Fratelli et al. cultured the *C. tetani* Harvard-Caracas strain in fed-batch conditions. The authors evaluated the impact of different initial concentrations of casein and glucose in static bottles with pulsed glucose addition. The best culture conditions were obtained with an initial casein peptone concentration between 50 and 62.5 g/L and an initial glucose concentration from 0.75 to 1.25 g/L. The glucose concentration was then adjusted to 3 g/L by pulsed glucose addition at 16, 56 and 88 h of culture. These fed-batch cultures produced 60 Lf/mL tetanus toxin (compared to 15 Lf/mL in the reference batch cultures) (Fratelli et al., 2010).

Based on the previously performed cultures (Table 3), the process that produced the highest toxin concentration seemed to be the continuous cultivation mode, with the concentration reaching 120 Lf/mL in modified Massachusetts medium. The advantage of the continuous culture process is that the cells are maintained in the exponential growth phase.

5.5. Optimal pH for *Clostridium tetani* cultivation

In continuous culture, the optimal pH for toxin production was 7.8, with the ability to produce toxin from pH values of 6.2 to 8.8 (Zacharias and Bjorklund, 1968). However, it was recommended that cultivation be performed at pH 7.4 since a higher pH leads to iron precipitation, producing a black color that interferes with biomass determination. To better understand the effect of pH, toxin stability was evaluated at lower and higher pH values, but no toxin degradation was found at these extreme pH values. Therefore, the authors suggested that the pH value affected either some enzymes involved in toxin production or the release capacity of the membrane.

In batch cultures, pH is usually not regulated. The initial pH is commonly in the range 6.8–7.2. Demain et al. (2003) showed that the initial pH had an influence on the autolysis phase. The cell lysis percentage decreased with increasing initial pH values. The authors obtained the best toxin titer (67.5 Lf/mL) at an initial pH of 6.1, corresponding to a final pH of 7.75 (Demain and Fang, 2003).

6. Nutritional needs of *Clostridium tetani* for bacterial growth and toxin production

6.1. Histidine peptide consumption from complex amino acid sources

To determine the role of the consumption of amino acids released upon hydrolysis of peptides, Mueller et al. (1953) studied the effect of pancreatic digest of casein used for *C. tetani* Harvard strain cultures. The pancreatic digest of casein was fractionated into three parts (acidic, neutral and basic). They found that the omission of any one of these parts eliminated toxin production. Toxin production was also inhibited when one of the fractions was hydrolyzed by acid. They also learned that free histidine did not satisfy the histidine requirements. Histidine had to be present in peptide form, such as glycyl-histidine or acetyl-histidine, to start toxin production. Thus, Mueller's team suggested that this requirement for the peptide-bound form could also exist for serine, glutamic acid and aspartic acid (Mueller and Miller, 1953).

Mueller et al. (1956) ran further experiments to study the effect of histidine peptides on toxin production by substituting the histidine part of the basic fraction of the pancreatic digest of casein with synthetic peptides. Eight different histidine peptides were evaluated: i) glycyl-histidine was found to be the most effective histidine peptide, since its use produced as much toxin as the use of the whole pancreatic digest of casein (130 Lf/mL and 135-140 Lf/mL, respectively), followed by L-α-amino-n-butyryl-L-histidine; ii) β-L-aspartyl-L-histidine, acetyl-histidine and L-carnosine were efficient only at very high concentrations; and iii) anserine, 1-methyl-histidine, and 3-methyl-histidine resulted in no toxin production. They concluded that the structural specificity of the linkage of histidine was the stimulatory element. Moreover, free histidine still had to be part of the medium, but it contributed only to bacterial growth (Mueller and Miller, 1956).

High histidine peptidase activity was measured from the beginning of growth, whereas intracellular toxin production started only after 10 h of cultivation (Miller et al., 1960). The

enzyme was only synthesized in media containing histidine peptides, leading to enhanced toxin production. Moreover, Fe^{2+} stimulated peptidase activity. The histidine peptidase was able to hydrolyze glycyl-L-histidine and L-α-amino-n-butyryl-L-histidine. The hydrolysis of acetyl-histidine was weaker and needed a high concentration of this peptide, corresponding to the observation already made by Mueller and Miller (Mueller and Miller, 1956). The peptidase activity was the same in cell-free assays. Therefore, the superiority of glycyl-histidine as a peptidase substrate or toxin synthesis effector was not dependent on its membrane transport. The authors demonstrated that toxin production was directly proportional to histidine peptide hydrolysis. They also checked that the toxin and the histidine peptidase were not the same protein (Miller et al., 1960). Later, Helting et al. found this enzyme in the supernatant and confirmed that it was active toward glycyl-histidine and not responsible for the proteolysis to a mature toxin (Helting et al., 1979).

6.2. Casein peptide consumption from complex amino acid sources

To further evaluate the impact of the casein pancreatic digest (NZ-Case) and identify the casein peptides with an effect on toxin synthesis, cultivation was performed for NZ-Case fractions with a Harvard strain in modified Massachusetts medium (supplemented with 1.01 g/L KH_2PO_4, 0.72 g/L $CaCl_2$ and 0.36 g/L active charcoal). Porfirio et al. (1997) found that only the omission of the neutral fraction of the casein digest decreased toxin production, by approximately 30 %, suggesting that this fraction contains one or several elements promoting toxin synthesis. The omission of the acidic or the basic fraction of the casein digest did not affect the toxin titer. Hence, they purified peptides from the neutral part and individually added them to the Massachusetts medium. They showed that the following peptides were the most active in toxin production, with a final toxin titer reaching 200 % to 265 % of the toxin production in Massachusetts medium:

- Ile – Pro – Ile – Gln – Tyr – Val
- Val – Leu – Gly – Pro – Val
- Ala – Val – Pro – Tyr – Pro – Gln
- Asp – Met – Pro – Ile
- Val – Ala – Pro – Phe – Pro – Glu – Val – Phe
- Glu – Met – Pro – Phe – Pro – Lys

They also pointed out that the glycyl-L-histidine peptide from the basic part of the casein digest favored toxin synthesis.

Zinc proteases are found in casein the tryptic digest, which is a component of Massachusetts medium (Table 2). The pattern Pro-hydrophobic-Pro is characteristic of several zinc peptidase inhibitors, and the tetanus toxin is a zinc protein. Thus, the authors suggested that the presence of these Pro-hydrophobic-Pro peptides could increase toxin production by protecting the toxin from proteolytic degradation. They also proposed that these peptide patterns could act as peptide-hormone signals in bacteria for toxin synthesis (Porfirio et al., 1997).

Licona-Cassani et al. (2016) identified specific peptides from casein digestion (NZ-Case) that were specifically consumed by *C tetani* E88. The authors pointed out that peptides were consumed after exhaustion of free amino acids. They suggested that this peptide consumption could explain the decrease in the specific growth rate, as peptide transport requires more energy than free amino acid transport. The consumed peptides presented the following specific shared patterns:

- Val – Pro – Gln – Leu – Glu – Ile – Val
- Val – Tyr – Pro – Phe – Pro – Gly – Pro – Ile

The last peptide harbors the Pro-Phe-Pro pattern, which was described by Porfirio *et al.* (1997) as a zinc protease inhibitor.

Licona-Cassani et al. (2016) also noticed that peptide consumption was related to *tetX* expression in a transcriptional analysis, concluding that these specific peptides were essential for activation of the production of tetanus toxin (Licona-Cassani et al., 2016).

Furthermore, toxin synthesis has been shown to depend on the content of Maillard reaction products. When the medium was sterilized by filtration or autoclaved for too long, no toxin was detected. The production of toxin required an adequate amount of Maillard reaction products, produced from the peptide–sugar reaction under heat (Chung et al., 2016).

Considering the studies on the role of peptides in toxin production, it seems that glycyl-histidine and the Pro-Phe-Pro peptide pattern have a significant effect on toxin production. They could be used to design a chemically defined medium promoting toxin synthesis.

6.3. Free amino acid consumption for *C. tetani* growth and toxin production

Despite the influence of peptides in toxin production, studies were conducted to design a chemically defined medium that could support *C. tetani* growth and toxin production. Two different media were evaluated (Table 4-A) (Feeney et al., 1943b; Licona-Cassani et al., 2016)). Glucose was present at the same concentration in both media, but the initial

compositions and concentrations of amino acids were not identical: i) Licona-Cassani et al. added L enantiomers of alanine, glutamine, glycine and (hydroxy)proline, which were not used by Feeney et al.; ii) Feeney et al. used L and D enantiomers or a racemic mixture, and iii) amino acids were more concentrated in Feeney et al. cultures. Salts and vitamins were also present but are not shown in the table. The medium used by Licona-Cassani et al. was based on the chemically defined medium used for a pathogenic streptococcus culture. The results of these two studies on free amino acid consumption are compiled in Table 4-B.

Feeney *et al.* (1943) analyzed the effects of the amino acids in chemically defined medium on growth by one-at-a-time omission (Feeney et al., 1943b). Yu *et al*. (2009) identified possible auxotrophy resulting from the lack of an amino acid biosynthesis pathway in *C. tetani* by bioinformatics analysis of its genome (Yu et al., 2009). Licona-Cassani *et al.* (2016) followed the amino acid consumption kinetics in both complex and chemically defined media (Licona-Cassani et al., 2016). No toxin production was observed on either chemically defined medium, but the analysis of the amino acids sustaining growth was convenient, as the media did not contain any complex materials.

Table 41-A: Initial glucose and amino acid compositions of chemically defined media

Glucose and amino acid components	**Feeney *et al*. (1943)**	**Licona-Cassani *et al*. (2016)**
	Amount (g) for 1 L	
Glucose	10	10
Alanine	-	0.1 (L-)
Arginine	0.5 (L-)	0.1 (L-)
Aspartic acid	0.2 (DL-)	0.1 (L-)
Cysteine	-	0.5 (L-)
Cystine	0.4 (L-)	0.05 (L-)
Glutamic acid	2.5 (D-)	0.1 (L-)
Glutamine	-	0.2 (L-)
Glycine	-	0.1 (L-)
Histidine	0.5 (L-)	0.1 (L-)
Isoleucine	0.3 (DL-)	0.1 (L-)
Leucine	0.3 (DL-)	0.1 (L-)
Lysine	0.2 (DL-)	0.1 (L-)
Methionine	0.2 (DL-)	0.1 (L-)
Phenylalanine	0.2 (DL-)	0.1 (L-)
Proline	-	0.1 (L-)
Hydroxy-Proline	-	0.1 (L-)
Serine	0.2 (DL-)	0.1 (L-)
Threonine	0.2 (DL-)	0.2 (L-)

Tryptophan	0.05 (L-)	0.1 (L-)
Tyrosine	0.3 (L-)	0.1 (L-)
Valine	0.3 (DL-)	0.1 (L-)

627 *(L-): L enantiomer; (D-): D enantiomer; (DL-): racemic mixture*

628 **Table 4-B: Free amino acids involved in *Clostridium tetani* growth**

Amino acids used for bacterial growth	**Feeney *et al*., 1943** **Harvard strain** **On chemically defined medium (Table 4-A)**	**Yu *et al*., 2009** **E88 strain [1]** **Deficient in amino acid biosynthetic pathways**	**Licona-Cassani *et al*., 2016** **E88 strain** **Approximate consumption**	
			On modified Massachusetts medium [2]	On chemically defined medium (Table 4-A)
Arginine	essential	-	0 %	20 %
Asparagine	not experimented	-	90 %	100 %
Aspartate	less effective	-	100 %	100 %
Cysteine	not experimented	-	90 %	not analyzed
Glutamate	stimulated	-	> 95 %	100 %
Glutamine	not experimented	-	90 %	100 %
Histidine	essential	auxotrophic	> 95 %	100 %
Isoleucine	essential	auxotrophic	0 %	15 %
Leucine	essential	auxotrophic	0 %	60 %
Lysine	less effective	auxotrophic	0 %	25 %
Methionine	less effective	auxotrophic	75 %	100 %
Phenylalanine	less effective	auxotrophic	0 %	20 %
Serine	stimulated	-	> 95 %	100 %
Threonine	stimulated	-	> 95 %	100 %
Tryptophan	essential [3]	auxotrophic	0 %	0 %
Tyrosine	essential	-	100 %	60 %
Valine	essential	auxotrophic	0 %	0 %
Growth	X_{max} = 80-85 % of the X_{max} obtained in Mueller and Miller medium	-	$\mu = 0.24\ h^{-1}$ during exponential growth, then $\mu = 0.083\ h^{-1}$ during slower growth	$\mu = 0.69\ h^{-1}$

629 *[1] Strain not specified; probably E88, as that was the only one sequenced at that time*

630 *[2] Massachusetts medium supplemented with cystine, uracil and vitamins and with trace*

631 *amounts of $FeCl_3$*

[3] *Tryptophan was not tested in this chemically defined medium but was identified as being essential in a previous experiment in complex medium* (Mueller & Miller, 1942).

Essential: no growth when the amino acid was omitted. Stimulated: little growth after 48 h of culture when the amino acid was omitted. Less effective: Good growth after 48 h of culture when the amino acid was omitted.

X_{max}*: maximum biomass concentration;* μ*: specific growth rate*

Among the eight putative auxotrophic amino acids (Yu et al., 2009), only five (His, Ile, Leu, Trp, Val) were considered essential by Feeney et al. (Table 4-B). The authors also identified arginine, isoleucine, tryptophan and valine as being essential for *C. tetani* growth.

Mueller and Miller (1949) pointed out that addition of glutamine could partially replace casein digestion. Using the Harvard strain in test tubes, a lowered casein content in the medium resulted in 3-fold lower toxin production. The addition of 0.25 g/L glutamine recovered half of the usual toxin titer (50 Lf/mL with glutamine to 90 Lf/mL for the control tube) (Mueller and Miller, 1949). This amino acid was totally consumed in the chemically defined medium described by Licona-Cassani (Licona-Cassani et al., 2016).

Among the amino acids potentially identified as auxotrophic by Yu et al. (2009), only histidine and leucine were significantly consumed in the culture grown in Licona-Cassani chemically defined medium (Table 4-B). Arginine, isoleucine, tryptophan and valine (identified as essential by Feeney *et al.*) were slightly or not consumed in the Licona-Cassani experiment in both complex and chemically defined media. Therefore, one can wonder if these amino acids are required for *C. tetani* growth. Growth stopped after the depletion of five amino acids (Glu, Ser, Gln, His, Thr), suggesting possible new auxotrophies for glutamate, serine, glutamine and threonine. By the end of the stationary phase, three other amino acids were completely consumed (Asp, Asn, Met). Overall, *C. tetani* assimilated thirteen amino acids to perform growth until a maximal optical density (530 nm) of 1.3 at a specific growth rate of $\mu = 0.69\ h^{-1}$ (Figure 5). From the observations in these two experiments in chemically defined medium and in combination with casein peptide studies, histidine seems to be of major importance for *C. tetani* growth.

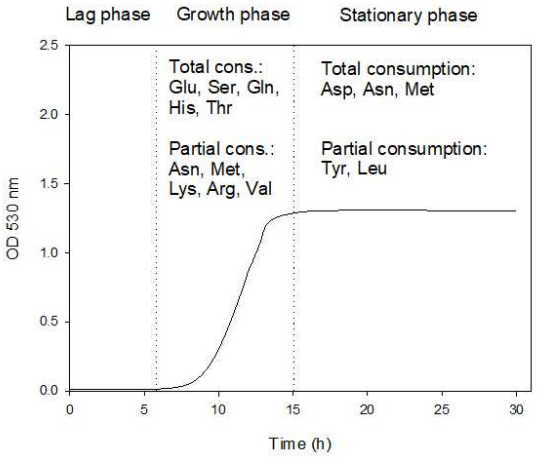

Figure 5: Amino acid consumption in chemically defined medium, ***adapted from Licona-Cassani et al. (2016)***

Licona-Cassani et al. (2016) observed in a modified Massachusetts medium that the depletion of free amino acids was correlated with the beginning of the slower growth phase when peptides started to be consumed. On the chemically defined medium, no toxin production was observed. As *tetX* expression is triggered when metabolism changes from amino acid to peptide consumption, tetanus toxin production might be caused by this metabolic transition. In the culture performed in a modified Massachusetts medium, the genes encoding the degradation of glutamate, histidine, serine, threonine and aspartate were found to be highly expressed during the exponential and the slower growth phases, showing the strong consumption of these amino acids (Licona-Cassani et al., 2016). In further experiments, these five amino acids were supplemented in the modified Massachusetts medium (+ 0.8 g/L of each amino acid) (Orellana et al. 2020). This resulted in a 2.5-fold increase in cell density, with a longer exponential growth phase (8.5 h instead of 5 h, with the same growth rate (0.46 h^{-1}) as that in the control experiment). The slower growth phase showed a twofold increase in growth rate with the addition of amino acids (0.145 h^{-1} instead of 0.06 h^{-1} for the control culture); however, the final toxin titer was twofold lower. Moreover, the genes involved in the aspartate degradation pathway for pyrimidine metabolism (CTC2383-2384) were expressed during the exponential growth phase, rather than during the slower growth phase, as observed in the control culture. Therefore, aspartate utilization in the pyrimidine pathway could be related to toxin regulation. More generally, the expression levels of the genes encoding glutamate, histidine, threonine, methionine and aspartate degradation differed in the two cultures, with stronger gene expression observed in the first hours of growth in the amino acid-supplemented culture. The expression of the genes related to serine and tyrosine metabolism was similar in the two conditions. The authors suggested that amino acid

supplementation could result in amino acid overflow, leading to the accumulation of metabolic intermediates or by-products, which could inhibit *tetR* transcription (Orellana et al., 2020). The studies presented in Table 4-B do not provide information on the effect of cysteine on the *C. tetani* growth requirements. In complex medium, addition of cystine (Cys-Cys peptide) after autoclaving instead of before autoclaving decreased toxin production, probably because of the effect of heat on cystine. Moreover, cystine inhibited toxin synthesis when added at a concentration of more than 125 mg/L (Latham et al., 1962).

6.4. Glucose consumption during *C. tetani* cultivation

In 1943, Mueller et al. reported that growth and toxin production occurred on complex medium with the omission of either glucose or cystine, but growth failed if both were omitted. Then, they suggested that both components might be involved in the maintenance of anaerobic conditions (Mueller et al., 1943).

On casamino acid semisynthetic medium, Kaufman et al. (1958) showed on four strains from the University of Kentucky laboratories that glucose was nonessential and nonstimulatory for *C. tetani* growth. However, on a chemically defined medium, glucose was nonessential for half of the strains but stimulatory for the other strains: with glucose omission, two strains exhibited normal growth (51 % light transmission), whereas the other two showed poorer growth (87 % light transmission). The authors suggested that glucose degradation compounds might be involved in some amino acid utilization processes. No toxin production was observed in this study (Kaufman and Humphries, 1958).

Martinez et al. (1959) showed that glucose was consumed only after 24 to 30 h of growth on a complex medium containing casein, yeast extract and glucose. From this time point, growth increased proportionally to glucose addition in the medium. By observing glucose phosphorylation in cell-free extract, the authors then confirmed the presence of a glucokinase in *C. tetani* Harvard and 45e strains. This phenomenon was specific to glucose, as other hexoses were not affected. They suggested that this enzyme was inducible because it showed no activity in cells grown in a medium without glucose (Martinez and Rittenberg, 1959).

In continuous culture on complex medium (Harvard derivative strain), regardless of whether the dilution rate or the initial glucose concentration was changed, no toxin was found in the supernatant until glucose was completely consumed. The toxin release was then related to glucose limitation (Zacharias and Bjorklund, 1968).

Fratelli et al. (2005) studied glucose requirements when a Harvard variant strain was cultured with an NZ-Case TT nitrogen source. They showed that the optimal glucose concentration was 9.7 g/L for 43.5 g/L NZ. With a low glucose concentration (2.3 g/L), growth occurred but did not lead to toxin production in the supernatant. In addition to optimizing the glucose concentration, they revealed that the ratio between carbon and nitrogen sources was a key factor. They established a relationship between glucose and nitrogen source described by the following formula, where G_0 (g/L) is the initial glucose concentration, and NZ_0 (g/L) is the initial casein digest concentration (Fratelli et al., 2005):

$$\text{Toxin (Lf/mL)} = 46.29 + 5.55\left(\frac{G_0}{4} - 2\right) + 26.82\,(\frac{NZ_0}{12.5} - 2) - 6.39\,(\frac{G_0}{4} - 2)^2 - 9.04\,(\frac{NZ_0}{12.5} - 2)^2$$

($p < 0.0001$; $R^2 = 0.76$)

On chemically defined medium containing an initial glucose concentration of 10 g/L, no glucose was consumed during growth (Licona-Cassani et al., 2016).

6.5. Effects of inorganic compounds on *C. tetani* growth and toxin production

6.5.1 Iron

The role of some growth factors can depend on other medium components. Lerner et al. (1949) related iron concentration to glucose consumption in Mueller and Miller medium. Cells in iron-deficient medium were unable to ferment glucose. Glucose consumption was semilogarithmically proportional to iron content and was estimated through carbon dioxide production. They suggested that an enzyme or coenzyme of the glucose pathway was iron dependent. Later, this enzyme was identified as pyruvate:ferredoxin oxidoreductase, containing Fe-S clusters, which catalyzes pyruvate degradation to acetyl-CoA (Bruggemann and Gottschalk, 2004). This enzyme needs oxidized ferredoxin as a substrate and releases it in its reduced form. Lerner *et al.* (1949) also experimented with glutamine addition in iron-deficient complex medium. This resulted in stimulated carbon dioxide production, approaching the production levels observed in high-iron medium. Thus, addition of glutamine could overcome iron deficiency (Lerner and Mueller, 1949).

According to Feeney et al. (1943), on casein hydrolysate medium, the optimum iron concentration was 50 mg/L reduced iron (powder) (Feeney et al., 1943a) or 0.3 mg/L $FeSO_4^-$

747 .7H_2O (Mueller and Miller, 1954). In both studies, at concentrations above these values,
748 growth was better, but toxin production decreased. Both growth and toxin production
749 therefore were dependent on iron content.

750 In an initial study, Demain et al. (2003) observed that reduced powdered iron content affected
751 the toxin titer only if the iron was autoclaved with the other compounds in Mueller and Miller
752 medium. When the iron was autoclaved separately, its concentration had no influence on toxin
753 production (Demain and Fang, 2003).

754 In 2006, when a Harvard derivative strain (from Wyeth-Lederle Vaccine and Pediatrics) was
755 cultured in soy-based medium, Demain *et al.* showed that iron was essential for toxin
756 production. However, only reduced iron powder yielded satisfactory toxin titers (56-73
757 Lf/mL) at an optimal concentration of 0.5 g/L. When the insoluble iron was replaced by a
758 soluble iron source such as ferric citrate, ferric gluconate or ferrous ammonium sulfate,
759 moderate toxin production (36-43 Lf/mL) was observed. Ferrous sulfate, ferric chloride and
760 ferric nitrate failed to support toxin production. However, supplementation of activated
761 charcoal with soluble iron sources (ferrous sulfate, ferric citrate, ferrous gluconate) increased
762 toxin production (53-68 Lf/mL), which was consistent with the result obtained with iron
763 powder. Charcoal provides an insoluble surface of growth for bacteria. When the insoluble
764 parts of the medium were removed by filtration, toxin production was almost halved. It seems
765 that solid iron could provide a surface for growth or could absorb some inhibitory compounds.
766 Iron in its solid form is also known to absorb oxygen, which is propitious for anaerobic
767 bacteria (Demain et al., 2006).

768 Licona-Cassani et al. (2016) observed no change in total soluble iron content (Fe^{2+} and Fe^{3+})
769 in a modified Massachusetts medium during fermentation, but this conclusion could be biased
770 because iron seemed to be present in excess in the medium (0.03 mg/L of $FeCl_3$), even though
771 it was diluted 1000x compared to the level in the initial Massachusetts medium (32 mg/L of
772 $FeCl_3$.6 H_2O). They determined that the total intracellular iron (Fe^{2+} and Fe^{3+}) content
773 decreased slightly during glucose metabolism, which was in accordance with the observation
774 of Lerner et al. (1949) that related iron concentration and glucose consumption. Moreover, the
775 transcriptomic analysis of E88 culture showed that Fe^{2+} transport genes (CTC451-452,
776 CTC534) were expressed in both growth phases, whereas Fe^{3+} di-citrate transport genes
777 (CTC784, CTC956, CTC961, CTC1371) were expressed during the slow growth phase, when
778 toxin was produced (Licona-Cassani et al., 2016).

779

6.5.2 Mineral salts

Mueller et al., 1943 reported that potassium, magnesium and phosphate inorganic salts were required for *C. tetani* growth (Mueller et al., 1943). In continuous culture, Zacharias and Björklund (1968) showed that the addition of 0.1 g/L potassium chloride in modified Massachusetts medium doubled the production of toxin (70 Lf/mL to 130 Lf/mL) in a Harvard strain derivative (strain 107). They pointed out that potassium has the property of increasing the permeability of the cell membrane. They observed the opposite phenomenon (70 Lf/mL to 40 LF/mL) with the addition of 0.15 g/L calcium chloride, which is known to reduce cell membrane permeability (Zacharias and Bjorklund, 1968). In TGY flask cultures of strain CN655, the optimal inorganic phosphate concentration for toxin production was found to be 40 mM (two-fold the extracellular toxin concentration in comparison to TGY without phosphate addition). Phosphate supplementation increased the transcription of *tetX* but not that of its transcriptional regulator *tetR*. Thus, the authors suggested that toxin regulation by phosphate is not related to TetR (Chapeton-Montes et al., 2020).

6.5.3 Vitamins

Vitamins and trace elements used to be supplied by beef heart infusion into Mueller and Miller medium for *C. tetani* growth. Latham et al. (1962) showed that beef heart infusion was not essential for growth and toxin production. Beef heart infusion can successfully be substituted with nicotinic acid, vitamin B12 and iron (Latham et al., 1962). When Hy-Soy® peptone was used, vitamin addition was not required. Hy-Soy® peptone may contain enough uracil, calcium pantothenate, thiamine, riboflavin, pyridoxal and biotin for *C. tetani* growth and toxin production. This complex material also satisfied the tyrosine and cystine requirements, as supplementation of these materials was no longer required (Demain et al., 2007).

In chemically defined medium, Feeney et al. (1943) showed that ten vitamins and assimilated compounds were essential for Harvard *C. tetani* growth: biotin, calcium pantothenate, folic acid, nicotinic acid, oleic acid, riboflavin, pyridoxine, thiamin, adenine and uracil (Feeney et al., 1943b).

The chemically defined medium used by Licona-Cassani et al. (2016) contained all of Feeney's vitamins and assimilated compounds except for oleic acid, which supported growth. On complex media, they observed that calcium pantothenate and riboflavin were consumed during amino acid metabolism, whereas the concentrations of pyridoxine and nicotinic acid

increased during fermentation. They also discovered that uracil was both taken up (amino acid metabolism) and released (autolysis) during cultivation (Licona-Cassani et al., 2016).

7. Conclusion

The *C. tetani* genome is highly conserved among all the sequenced strains, especially among strains used for toxin production (Harvard strains, clade 1A). Notably, the toxin-encoding genes show 99.3–99.4 % sequence identity. This identity reached 100 % for the transcriptional regulator gene sequence. Whole-genome sequencing and determination of *C. tetani* metabolic pathways showed a metabolism oriented toward amino acid assimilation, with many enzymes dedicated to the transport and degradation of amino acids, which is common for pathogenic bacteria. Even though this microorganism has been used for many years, the regulators that induce the regulatory system for toxin synthesis have not been fully identified. Several experimental strategies have led to the development of different chemically defined media that are able to sustain *C. tetani* growth, but none of these strategies have led to toxin production. For now, peptides appear to be essential for induction of toxin production. In particular, histidine seems to be of major importance in *C. tetani* growth and toxin production, either in its free or peptide form. The role of glucose in *C. tetani* toxin production is still unclear. It might contribute to toxin synthesis, for example, by generating metabolites of interest or by an unknown interaction. Iron appeared to enhance toxin production depending on form in which it was supplied.

This overview of the metabolism combined with the previous nutritional studies for *C. tetani* growth and toxin synthesis gathered in this review could provide support for the design of new experiments on growth and toxin production in chemically defined media. For instance, cultivation with regular sampling and an in-depth analytical strategy (including substrate consumption and metabolite determination) could provide new knowledge on specific features of *C. tetani*. Combining transcriptomic and proteomic analyses within controlled fermentation systems should provide a better understanding of global metabolism and could help determine the key compounds involved in *C. tetani* growth and toxin synthesis. These experiments could help to provide a new chemically defined medium that would support both growth and tetanus toxin synthesis and would thus satisfy the World Health Organization recommendations for tetanus vaccine production.

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Conflicts of interest

The authors declare no conflicts of interest.

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