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# 1            Insights into *Clostridium tetani*: from genome to bioreactors

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7    Keywords: *Clostridium tetani* culture, tetanus toxin production, central metabolism map,  
8    nutritional requirements, chemically defined medium

## 9            **Abstract**

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

## 27 **1. Introduction**

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

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

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

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

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

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

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

## 71 **2. *Clostridium tetani* genome and central metabolism**

### 72 **2.1. *Clostridium tetani* strain diversity and genome sequences**

73 To date, 43 *C. tetani* strains have been isolated, mainly from human wounds, in different  
74 geographic locations worldwide (North America, China, France) (Chapeton-Montes et al.,  
75 2019; Cohen et al., 2017; U.S. National Library of Medicine). Strains are classified into two  
76 main groups: the Harvard strains derived from the ancestral Harvard strain (Clade 1A) and the  
77 wild-type strains isolated from clinical cases (Clades 1B-H and 2). The ancestral Harvard  
78 strain was collected by the Hygienic Laboratory of the US Public Health Service by the end of  
79 the First World War. That strain was then distributed to laboratories and vaccine  
80 manufacturers from the 1920s to the 1950s. This led to the development of the Harvard family  
81 strains, which are characterized by low sporulation and high toxin production and are suitable  
82 for vaccine production. The strains E88 and CN655, isolated from the Harvard family, are  
83 commonly used in European laboratories.

84 The 2.8 Mb genome of the *C. tetani* reference strain was sequenced in 2003 by Bruggemann  
85 (Bruggemann et al., 2003). The genome of *C. tetani* strain Harvard E88 encodes 2,372 ORFs  
86 (28.6 % G + C content) and a unique 74-kbp plasmid (24.5 % G + C content) with 61 ORFs.  
87 That plasmid includes the gene encoding the tetanus toxin (*tent*), its transcriptional regulator  
88 *tetR*, and genes encoding virulence factors (surface layer and adhesion proteins, collagenase).  
89 On the plasmid, ABC transporter-encoding genes (CTP24-25) were found directly

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

96 Among the 20 genes annotated as surface layer proteins in the E88 genome, a surface layer  
97 protein encoded by the chromosomal *slpA* gene (CTC462) was characterized by Qazi et al.  
98 (2007). The results showed a variability in its molecular weight depending on the strain (160  
99 kDa in CN655, 180 kDa in the clinical isolate NC06336-07, 160 to 180 kDa in the three  
100 clinical isolates CTHCM 19, 22 and 25) compared to a 118 kDa predicted protein. No  
101 glycosylation was found to explain this variability (Qazi et al., 2007). Such observations were  
102 already described by Takumi et al. (1991) with the characterization of the protein from the  
103 AO174 strain with a molecular weight of 140 kDa. Takumi et al. (1991) found that SlpA had  
104 an unusually low proline content and showed various isoelectric forms from pH 4.0 to 4.5.  
105 This surface layer protein presented an antigen, making immunological detection possible  
106 (Takumi et al., 1991).

107 Brüggemann et al. (2004) identified a heme oxygenase (CTC2478) in *C. tetani* that could  
108 confer oxygen tolerance to bacteria during wound infection by creating a local anaerobic  
109 environment. This enzyme was also found in another *Clostridium* wound colonizer  
110 (*Clostridium perfringens*) but not in the other pathogenic or nonpathogenic *Clostridia*. Thus,  
111 *C. tetani* is an anaerobic microorganism but is reported as an aerotolerant species, similar to  
112 *C. perfringens* (Bruggemann et al., 2004). Dedic et al. (1956) succeeded in growing *C. tetani*  
113 under aerobic conditions by the addition of cobalt (as  $\text{Co}(\text{NO}_3)_2$ ). Under anaerobic conditions,  
114 cobalt supplementation did not influence growth. The authors suggested that cobalt addition  
115 reduced the oxygen partial pressure of the cultivation medium (Dedic and Koch, 1956);  
116 however, this observation could also be related to the presence of heme oxygenase.

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

123 by CTC1019-1024) that transports the sodium ions out and regenerates NADH and oxidized  
124 ferredoxin. This sodium flow could then potentially be used to conserve energy through a V-  
125 type sodium ATP synthase (CTC2326-2332), producing ATP (Bruggemann and Gottschalk,  
126 2004). E88 also harbors the gene encoding the CodY protein (CTC1260), which is a global  
127 regulator. This protein is known to indirectly downregulate toxin production in *Clostridium*  
128 *difficile* (Dineen et al., 2007) but positively regulate toxin synthesis in *C. perfringens* (Li et  
129 al., 2013) and *Clostridium botulinum* (Zhang et al., 2014).

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

134 To date, the whole-genome sequences of the 43 isolated strains have been reported (U.S.  
135 National Library of Medicine). All the genome sequences were compared to the genome of  
136 the model strain E88. Analysis showed that genetic identity among variants was high. The  
137 toxin-encoding genes have similar sequences with 99.3–99.4 % identity among all Harvard  
138 strains harboring the tetanus plasmid (two strains lost their plasmid and thus their  
139 pathogenicity). These Harvard and wild-type strains also harbor an identical toxin  
140 transcriptional regulator gene sequence with 100 % identity (Cohen et al., 2017). Strain-  
141 specific proteins are encoded in their prophage regions. Other differences among these strains  
142 are found in their CRISPR/Cas loci and spacer regions. They mainly encode a set of fitness  
143 functions that protect these strains from environmental stress or new prophage infections  
144 (Bruggemann et al., 2015). Among the 43 *C. tetani* genome sequences reported, Chapeton-  
145 Montes et al. (2019) analyzed 38 genome sequences and showed that the core genome  
146 represents 77 % of the E88 genome. The 38 strains share 1,266 coding sequences (CDS; 32 %  
147 of the total CDS). The Harvard strains only differ by 292 single-nucleotide polymorphisms  
148 (SNPs) in their whole genomes and share a 100 % identical tetanus toxin at the protein level.  
149 They harbor strain-specific genes, encoding “a plasmid-like element carrying a toxin-antitoxin  
150 system, a gene cluster encoding surface-layer proteins, an iron transport system and a putative  
151 cell wall/spore coat/envelope/membrane modification system” (Chapeton-Montes et al.,  
152 2019). Phylogenetic analysis showed that the *C. tetani* plasmid evolved together with the  
153 chromosome and is not a recent genomic element. This reflects the genomic stability of *C.*  
154 *tetani* (Chapeton-Montes et al., 2019; Cohen et al., 2017). The *C. tetani* genome is considered  
155 to be stable in comparison with the genomes of other species, such as *C. botulinum*

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

## 159 **2.2. *Clostridium tetani* central metabolism**

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

176 As *C. tetani* assimilates many amino acids, its genome lacks biosynthetic pathways for at least  
177 phenylalanine, histidine, isoleucine, lysine, leucine, methionine, tryptophan and valine,  
178 causing amino acid auxotrophy. This is typical for pathogenic bacteria with small genomes:  
179 they do not develop pathways for amino acid biosynthesis because they live in a host (Yu et  
180 al., 2009). In addition to amino acid transport and degradation genes, *C. tetani* harbors  
181 numerous extracellular and intracellular peptidase- and (phospho)lipase-encoding genes. This  
182 set of peptidases, which are characteristic of pathogenic *Clostridia*, provides free amino acids  
183 to the bacteria. Genome sequence analysis data indicates that *C. tetani*, similar to *Clostridium*  
184 *butyricum*, could ferment glycerol to produce 1,3-propanediol (Gonzalez-Pajuelo et al., 2006)  
185 (glycerol kinases CTC1758, CTC2462; glycerol-3-phosphate dehydrogenases CTC596,  
186 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**

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

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

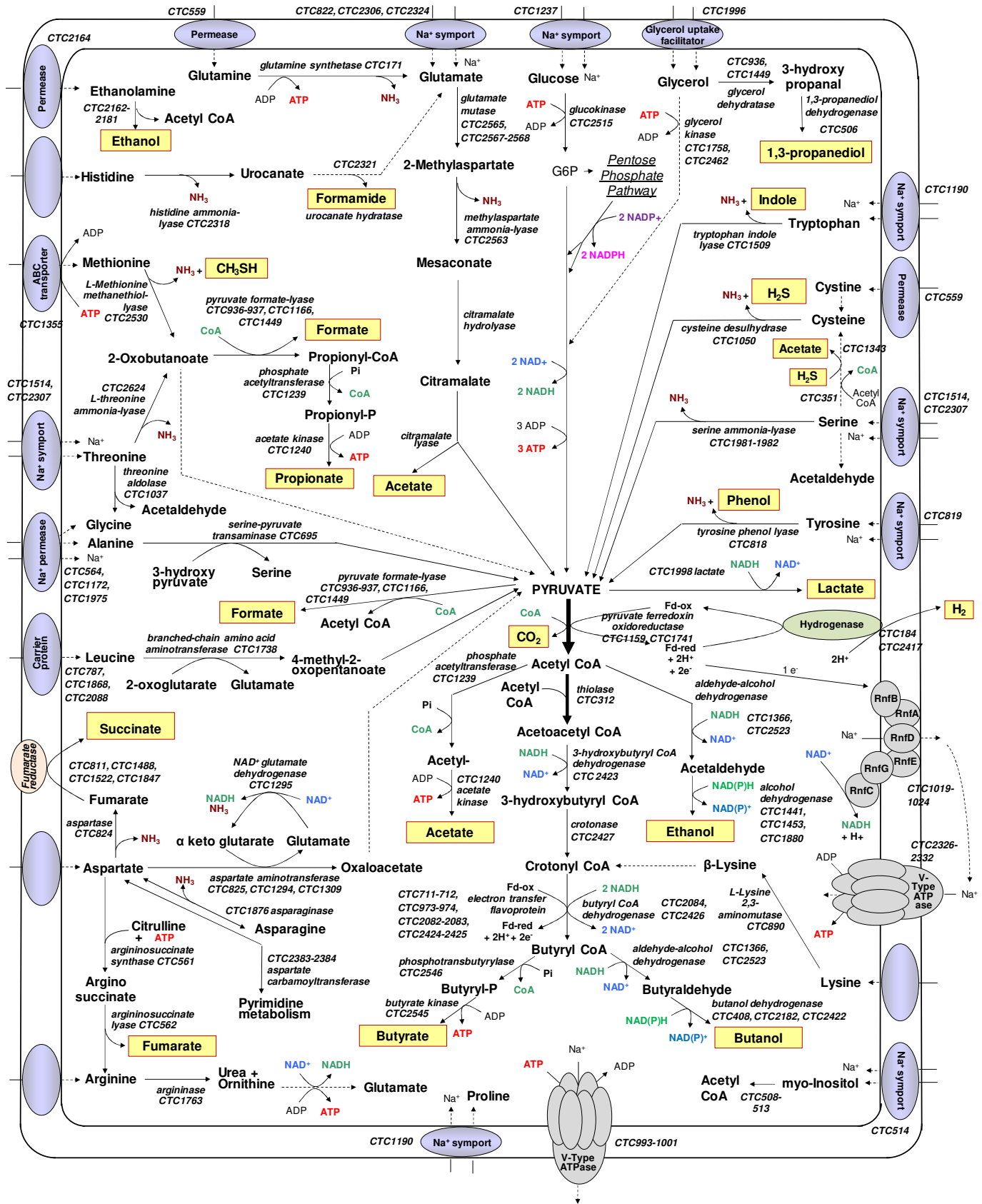
Amino acid	Major degradation pathways	Minor degradation pathways
<b>Aspartate</b>	Glutamate + Oxaloacetate	Ammonium + Fumarate
<b>Glutamate</b>	Acetate + Butyrate + Pyruvate + Ammonium	2-oxoglutarate + Ammonium n-Formimino-L-Glutamate
<b>Histidine</b>	Glutamate	Histamine
<b>Methionine</b>	2-Oxobutanoate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

197

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

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

218 from *C. acetobutylicum* and *C. kluyveri*, respectively, strongly suggesting its NADH and  
219 ferredoxin dependence in *C. tetani*. The Hbd enzyme was shown to be NADH dependent in  
220 *C. acetobutylicum* and NADPH dependent in *C. kluyveri* (Yoo et al., 2020). The Hbd  
221 CTC2423 from *C. tetani* has a higher sequence identity with *C. acetobutylicum* (80.5 %) than  
222 with *C. kluyveri* (68.9 %), suggesting that it is an NADH-dependent enzyme. Both the AdhE1  
223 and AdhE2 aldehyde-alcohol dehydrogenases from *C. acetobutylicum* were biochemically  
224 characterized *in vitro* and shown to be strictly NADH dependent (Yoo et al., 2015). Both  
225 enzymes have dual functionality, participating in the conversion of i) butyryl-CoA to  
226 butyraldehyde and ii) acetyl-CoA to acetaldehyde. A similar enzyme, CTC1366, sharing 68.2  
227 % amino acid identity with AdhE1 and 67.2 % amino acid identity with AdhE2, was  
228 identified in *C. tetani*. The three enzymes showed a conserved GCGXWG domain, which is  
229 commonly involved in coenzyme binding (Fontaine et al. 2002). Thus, on the basis of amino  
230 acid identity, aldehyde-alcohol dehydrogenase in *C. tetani* is likely NADH dependent.  
231 Finally, a butanol dehydrogenase, CTC408, catalyzing the conversion of butyraldehyde to  
232 butanol, was also identified in *C. tetani*. This enzyme shares 66.8 % amino acid identity with  
233 BdhA and 63.8 % amino acid identity with BdhB from *C. acetobutylicum*, which were  
234 biochemically demonstrated *in vitro* to be NADPH dependent (Yoo et al. 2015). Thus,  
235 butanol dehydrogenase in *C. tetani* could be considered to be NADPH dependent.  
236 Extrapolation to *C. tetani* based on the above % amino acid identity suggests that butanol  
237 production could be dependent on NADH and NADPH in *C. tetani* as well.



238  
 239  
 240

**Figure 1: *Clostridium tetani* central metabolism**  
 Yellow boxed compounds are fermentation products that are released in the medium

241 The general metabolic network in *C. tetani* is outlined in Figure 1. The enzymes involved in  
242 this metabolism are detailed with the number for the encoding gene. Their *in vivo*  
243 functionality has not been demonstrated. This metabolic map is not exhaustive. However,  
244 some uncertainties remain. Several amino acid transporters have not been identified (histidine,  
245 aspartate, arginine and lysine transporters). The glutamate/glutamine degradation pathway  
246 that functions through methylaspartate was first shown to be functional in *C. tetani* NCTC  
247 5404 using C-labeled glutamate (Buckel and Barker, 1974). In 2016, Licona-Cassani et al.,  
248 using in-depth transcriptional analysis, observed high expression of the methyl aspartate  
249 mutase cluster genes during the exponential phase, suggesting that glutamate is probably also  
250 metabolized via the mesaconate pathway in the E88 strain.

### 251 **2.3. *Clostridium tetani* bioenergetics**

252 Twenty-one iron-sulfur proteins with a [4Fe4S] core pattern and twenty-six homologous  
253 flavoproteins were found in *C. tetani*, suggesting a significant role of these electron transport  
254 proteins in metabolism. Among these iron-sulfur proteins, ferredoxins take part in electron  
255 transfer in *C. tetani*. Ferredoxin is reduced during the conversion of pyruvate to acetyl-CoA.  
256 Reoxidation of reduced ferredoxin generates an H<sup>+</sup> flow, which can be converted to  
257 dihydrogen by hydrogenases or used by ferredoxin NAD<sup>+</sup> oxidoreductases such as the Rnf  
258 complex. In addition, FeS clusters are found, in particular, in a membrane-bound system  
259 (CTC1019-1024) homologous to the Rnf complex (*Rhodobacter*-specific nitrogen fixation),  
260 described in acetogenic bacteria as a respiratory enzyme complex catalyzing the oxidation of  
261 reduced ferredoxin and the reduction of NAD<sup>+</sup>, generating a transmembrane ion gradient. In  
262 *Acetobacterium woodii*, the Rnf complex was proposed to be coupled to the translocation of  
263 sodium ions across the cytoplasmic membrane (Schuchmann and Muller, 2014). Thus, the  
264 Rnf-like system in *C. tetani* is suspected to maintain the sodium-motive force in the cell,  
265 which is essential for amino acid transport and for transport of electron flow from ferredoxin  
266 activity to regenerate the NADH pool. The (V)-type ATPases are the main ATPases in *C.*  
267 *tetani* (CTC993-1001, CTC2326-2332). The ATPase CTC993-1001 also participates in the  
268 transport of Na<sup>+</sup> ions out of the cell, reinforcing the sodium-motive force of the Rnf-like  
269 system. This sodium transport is then combined with energy dissipation. No F<sub>0</sub>-F<sub>1</sub>-type  
270 ATPase was found in *C. tetani*, which is unusual. Nonetheless, in contrast to other *Clostridia*,  
271 *C. tetani* harbors a gene cluster (CTC2326-2332) encoding a V-type sodium ATP synthase  
272 homologous to the archaeal-type ATP synthase (Bruggemann and Gottschalk, 2004). This

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

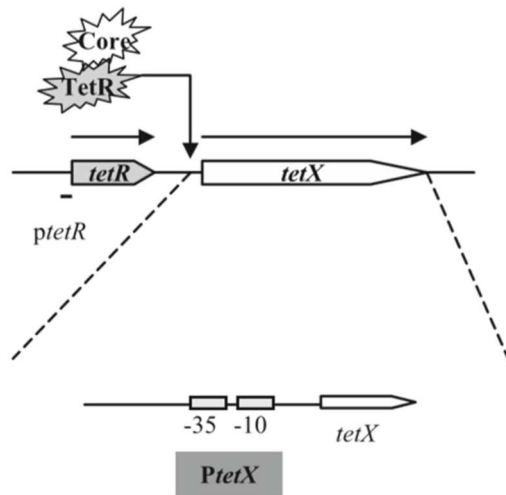
278 An H<sup>+</sup> pump (V-Type pyrophosphatase CTC383), coupled with the cleavage of inorganic  
279 pyrophosphate, was also found in *C. tetani*, which is uncommon in bacteria. In addition, a  
280 specific sodium ABC transporter (CTC1485) and several H<sup>+</sup>/Na<sup>+</sup> antiporters (CTC567,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

### 285 **3. Tetanus toxin regulation and synthesis**

#### 286 **3.1. Tetanus toxin regulation**

##### 287 **3.1.1 Toxin regulation by an alternative sigma factor**

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



296

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

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

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

### 307 **3.1.2. Toxin regulation by Two-Component Systems**

308 Tetanus toxin synthesis is regulated by a complex network of TCSs. For toxin synthesis, at  
 309 least two positive regulator TCSs and one negative regulator TCS were identified by  
 310 Chapeton-Montes *et al.* (2020). The first positive regulator TCS is located 25 kb upstream of  
 311 *tetX* on the plasmid, close to ATP-binding protein-encoding genes, but does not show any  
 312 homology with other bacteria. The other positive regulator TCS is located on the chromosome  
 313 and is putatively related to autolysis. These two TCSs are suspected to indirectly regulate  
 314 toxin synthesis since their proteins do not bind to TetR or TetX promoters. In contrast, the  
 315 negative regulator TCS directly represses toxin synthesis by binding to the TetR and TetX  
 316 promoters. On the basis of homology, this TCS, located on the chromosome, is involved in  
 317 cell division. Some other TCSs were suspected to act indirectly on toxin concentration by  
 318 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

319  
320 Chapeton-Montes et al. (2020) showed that toxin synthesis was also regulated by CodY. This  
321 protein, often involved in toxin and virulence regulation in other Gram-positive pathogenic  
322 bacteria, exhibited binding to the *tetX* promoter. This increased *tetX* transcription, which  
323 positively regulated toxin synthesis. As CodY is known to respond to starvation conditions,  
324 the authors suggested that this toxin regulation could be related to nutritional stress  
325 (Chapeton-Montes et al., 2020). Comparative transcriptional analysis of E88 grown in  
326 complex medium supplemented or not with an amino acid mixture suggested also that toxin  
327 production and virulence factors were related (Orellana et al., 2020). Specifically, the  
328 decrease in expression of virulence-related surface/cell-adhesion genes (CTC769-770,  
329 CTC772, CTC776-777) and flagellar genes considered as virulence-related genes in other  
330 pathogens (CTC1653-1679) affected toxin production. However, the genes encoding the  
331 homologous TCS of the virulence genes VirS/VirR in *C. perfringens* showed no difference in  
332 expression, regardless of the tetanus toxin production level.

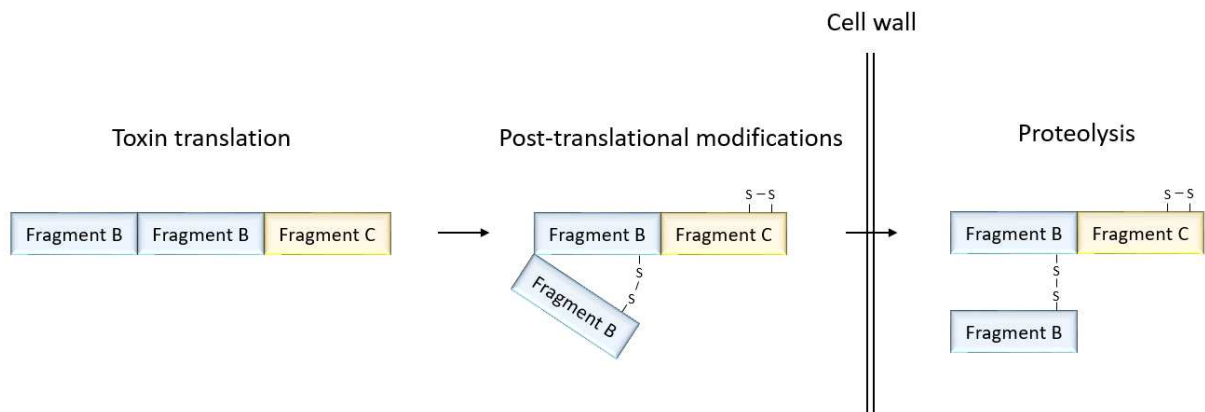
333 *C. tetani* phenotypes seemed to depend on toxin synthesis, with the long-chained filaments  
334 appearing in the culture with the highest toxin production (Orellana et al., 2020). Yet, the  
335 expression of cell division and cell wall elongation genes was not linked to toxin synthesis  
336 when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

### 3.2. Toxin production and maturation

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

343 After translation of the RNA to protein, the first methionine residue is removed, and two  
344 cysteine disulfide bridges are formed. The tetanus toxin is released in the culture medium.  
345 The toxin peptide chain is then hydrolyzed by a 27-kDa protease in the culture broth, resulting  
346 in a toxin composed of a 52-kDa light chain and a 98-kDa heavy chain (T. B. Helting et al.,  
347 1979). One disulfide bond links the heavy chain to the light chain, and another one forms a  
348 loop on the heavy chain. The single-chain toxin is cleaved between Glu449 and Asn450.  
349 However, toxins were also found in the culture broth as cleaved from Glu449 to Ser457,

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



352

353 **Figure 3: Maturation process of *Clostridium tetani* toxin**

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

361 In 1977, Helting et al. defined tetanus toxin as a two-fragment protein: fragment B (95-kDa,  
362 both on light and heavy chains) and fragment C (47-kDa, on heavy chain). Fragment C is the  
363 toxin part that triggers the strongest antigenic reaction, although it is not toxic (Helting et al.  
364 1977).

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

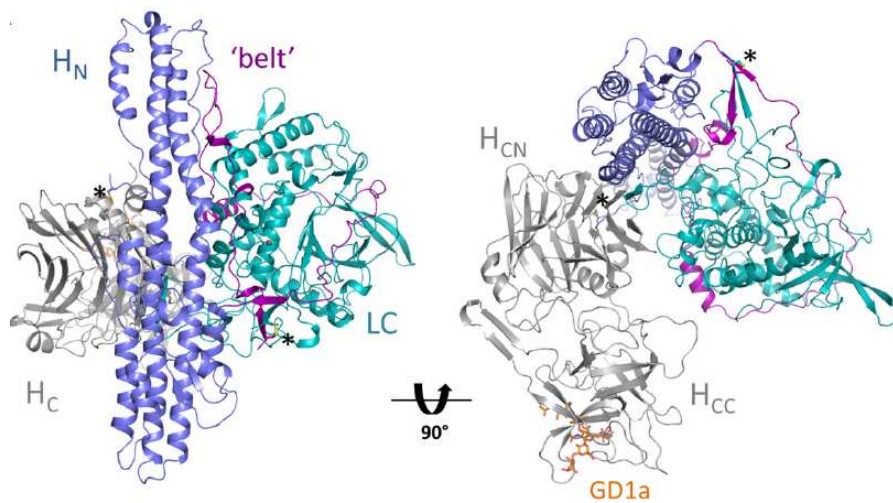
369 The tetanus toxin is very stable among the different strains because of its preserved amino  
370 acid sequence. Only a few changes in its amino acid composition were identified in clinical  
371 isolate strains. Its histidine pattern (HExxH) and its binding residues are always conserved  
372 (Chapeton-Montes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

373



374 **3.3. Toxin tridimensional structure**

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



383

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

385 *Green: light chain (LC). Blue: translocation domain (Licono-Cassani et al.). Gray: binding domain*  
386 *(H<sub>C</sub>). Pink: belt surrounding the light chain. Asterisk: disulfide bridges. Orange: GD1a ganglioside*  
387 *(receptor of tetanus toxin over the neurons)*

388

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

## 393 **4. Analytical methods for toxin detection**

### 394 **4.1. Flocculation method**

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

### 407 **4.2. Minimal lethal dose**

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

### 414 **4.3. Enzyme-linked immunosorbent assay**

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

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

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

## 429 **5. *Clostridium tetani* fermentation processes**

### 430 **5.1. Culture media for *Clostridium tetani***

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

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

<b>Constituents per liter</b>	<b>Final Mueller and Miller medium (Mueller and Miller, 1954)</b>	<b>Massachusetts medium (Latham et al., 1962)</b>	<b>Soy medium (Demain et al., 2005)</b>	<b>TGY medium (Chapeton-Montes et al., 2020)</b>
<b>Digest of casein</b>	22.5 g	25 g	-	30 g
<b>Beef heart infusion</b>	50 mL	-	-	-
<b>Yeast extract</b>	-	-	-	20 g
<b>Soy peptone</b>	-	-	34 g	-
<b>Glucose</b>	11 g	8.0 g	7.5 g	5 g
<b>NaCl</b>	2.5 g	2.5 g	5.0 g	-
<b>Na<sub>2</sub>HPO<sub>4</sub></b>	2 g	-	0.5 g	-
<b>KH<sub>2</sub>PO<sub>4</sub></b>	0.15 g	-	0.175 g	-
<b>MgSO<sub>4</sub></b>	0.15 g	0.1 g	0.024 g	-
<b>Cystine</b>	0.25 g	0.125 g	-	-
<b>Cysteine</b>	-	-	0.125 g	-
<b>Cysteine HCl</b>				0.5
<b>Tyrosine</b>	0.5 g	-	0.125 g	-
<b>Calcium pantothenate</b>	1.0 mg	1.0 mg	-	-
<b>Uracil</b>	2.5 mg	1.25 mg	-	-
<b>Nicotinic acid</b>	-	0.25 mg	-	-
<b>Thiamine</b>	0.25 mg	0.25 mg	-	-
<b>Riboflavin</b>	0.25 mg	0.25 mg	-	-
<b>Pyridoxine</b>	0.25 mg	0.25 mg	-	-
<b>Biotin</b>	2.5 µg	2.5 µg	-	-
<b>Vitamin B<sub>12</sub></b>	-	0.05 µg	-	-
<b>Reduced iron (powder)</b>	0.5 g	-	0.5 g	-
<b>FeCl<sub>3</sub> · 6 H<sub>2</sub>O</b>	-	32 mg	-	-
<b>pH adjustment (before autoclave)</b>	7.0 - 7.2	7.0 ± 0.2	6.8	7.5

447

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

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

454

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

Source	Strain	Cultivation mode and volume	Cultivation medium	Gas stripping	Growth	Toxin
<b>(Zacharias and Bjorklund, 1968)</b>	Strain 107 (Harvard strain derivative)	Bioreactor 1 L Continuous mode	Modified Massachusetts medium	N <sub>2</sub> gassing	0.125 h <sup>-1</sup> dilution rate	120 Lf/mL ± 10 Lf/mL
<b>(Jagicza et al., 1981)</b>	Harvard strain	Bioreactor 400 L Batch mode	Mueller and Miller medium	N <sub>2</sub> surface flushing for 16 h, then air flow	X <sub>max</sub> = 10 <sup>8</sup> cells/mL	55 Lf/mL
<b>(Gutiérrez et al., 2005)</b>	Massachusetts strain (Harvard strain derivative)	Bioreactor 5 L Batch mode	Modified Massachusetts medium	N <sub>2</sub> bubbling during expo. growth, then surface aeration	μ = 0.46 h <sup>-1</sup> X <sub>max</sub> = 0.78 g/L	73 Lf/mL
<b>(Demain et al., 2007)</b>	Wyeth-Lederle Vaccine and Pediatrics strain	Bioreactor 0.8 L Batch mode	Modified soy medium	N <sub>2</sub> bubbling	Not reported	48 Lf/ml
<b>(Fratelli et al., 2010)</b>	Harvard-Caracas (Harvard strain derivative)	Bottles 4 L Fed-batch mode	Modified Massachusetts medium	None	X <sub>max</sub> = 4.34 g/L	70 Lf/mL
<b>(Muniandi et al., 2013)</b>	Harvard strain derivative	Bioreactor 400 L Batch mode	Modified Mueller medium	Air surface flow	OD <sub>max</sub> = 2.3	70 Lf/mL
<b>(Licon-Cassani et al., 2016)</b>	E88	Bioreactors 1 L and 5 L Batch mode	Modified Massachusetts medium	N <sub>2</sub> surface flushing for 4 h, then air flow	Expo: μ = 0.24 h <sup>-1</sup> Slow: μ = 0.08 h <sup>-1</sup>	30.2 Lf/mL ± 2.9 Lf/mL
<b>(Chung et al., 2016)</b>	Harvard strain derivative	Bioreactor 50 L Batch mode	Modified Massachusetts medium	Punctual N <sub>2</sub> surface flow, then air flow	X <sub>max</sub> = 3 g/L	80 Lf/ml
<b>(Chawla et al., 2016)</b>	Harvard strain derivative	Bioreactor 1 L Batch mode	Modified Massachusetts medium with soy	Not reported	Not reported	97.9 ± 3.3 Lf/mL

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

## 458 **5.2. *Clostridium tetani* growth phases in batch mode**

459 *C. tetani* is mainly cultivated under batch mode in closed tubes or bottles. Only the end  
460 biomass and final toxin titer were reported, with no information on growth or toxin kinetics.  
461 Some bioreactor cultures were also described (Table 3). Jagicza et al. (1981) reported an  
462 industrial culture of the Harvard strain in Mueller and Miller medium. The biomass grew for  
463 three days from  $10^5$  to  $10^8$  cells/mL, with a pH decrease from 7.3 to 6.6. The toxin was  
464 intracellularly produced after one day of culture until the end of growth. The toxin was then  
465 released in the medium at a final toxin titer of 55 Lf/mL. A similar growth profile was also  
466 observed in a closed flask by Fratelli et al. (2005) with a maximal biomass concentration of  
467 4.4 g/L and a final toxin titer of 40 Lf/mL using the Harvard-Caracas strain grown in  
468 Massachusetts medium (Fratelli et al., 2005). In 2016, Licona-Cassani et al. reported batch  
469 cultivation of the E88 strain on Massachusetts medium supplemented with cystine, uracil,  
470 vitamins and traces amounts of  $FeCl_3$  (Table 3). During cultivation (in five independent  
471 experiments), regular sampling was applied to rigorously follow the growth of *C. tetani* in the  
472 bioreactor. Two distinct growth phases were observed: i) an exponential growth ( $\mu = 0.24 \text{ h}^{-1}$ )  
473 phase leading to a decrease in pH (from 6.8 to 6.6), lasting 10 h and exhibiting preferential  
474 consumption of free amino acids and ii) a slower growth ( $\mu = 0.083 \text{ h}^{-1}$ ) phase with an  
475 increase in pH (from 6.6 to 7.0), lasting 30 h, and associated with the consumption of peptides  
476 and glucose. The main virulence factor genes were activated, including *tetX*. The growth was  
477 then followed by a cell autolysis phase, lasting 30 h, with a decrease in optical density and an  
478 increase in pH (from 7.0 to 8.0). This autolysis appeared to be related to the physiological cell  
479 state. These five cultivations resulted in the production of  $30.2 \pm 2.86$  Lf/mL toxin (Licona-  
480 Cassani et al., 2016), which is lower than the values for all other batch cultures reported in the  
481 literature (Table 3).

## 482 **5.3. Continuous culture of *Clostridium tetani***

483 A continuous culture of a *C. tetani* Harvard derivative strain (strain 107) was reported in the  
484 literature. In 1968, Zacharias and Björklund studied the effects of different i) dilution rates  
485 ( $0.030 \text{ h}^{-1}$  to  $0.169 \text{ h}^{-1}$ ), ii) temperatures ( $32^\circ\text{C}$  to  $39^\circ\text{C}$ ), iii) pH values (5.9 to 9.5) and iv)  
486 salts (0.1 g/L KCl, 0.15 g/L  $CaCl_2$ ) in a modified Massachusetts medium (all concentrations

487 reduced by half). For each culture, one of these parameters was varied. The best toxin titer  
488 (120 Lf/mL toxin) was reached at a dilution rate of 0.125 h<sup>-1</sup>, a temperature of 34°C and a pH  
489 of 7.4 in the modified Massachusetts medium supplemented with 0.1 g/L of potassium  
490 chloride. Growth rates from 0.03 h<sup>-1</sup> to 0.125 h<sup>-1</sup> resulted in similar biomass and toxin  
491 production, with a biomass concentration of 0.7 g/L and a toxin titer of 70-75 Lf/mL.  
492 Potassium was used to increase membrane permeability and thus toxin release in the  
493 extracellular cultivation medium. The element controlling the growth at steady state was not  
494 described (Zacharias and Bjorklund, 1968).

#### 495 **5.4. Fed-batch culture of *Clostridium tetani***

496 In 2010, Fratelli et al. cultured the *C. tetani* Harvard-Caracas strain in fed-batch conditions.  
497 The authors evaluated the impact of different initial concentrations of casein and glucose in  
498 static bottles with pulsed glucose addition. The best culture conditions were obtained with an  
499 initial casein peptone concentration between 50 and 62.5 g/L and an initial glucose  
500 concentration from 0.75 to 1.25 g/L. The glucose concentration was then adjusted to 3 g/L by  
501 pulsed glucose addition at 16, 56 and 88 h of culture. These fed-batch cultures produced 60  
502 Lf/mL tetanus toxin (compared to 15 Lf/mL in the reference batch cultures) (Fratelli et al.,  
503 2010).

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

#### 508 **5.5. Optimal pH for *Clostridium tetani* cultivation**

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



517 In batch cultures, pH is usually not regulated. The initial pH is commonly in the range 6.8–  
518 7.2. Demain et al. (2003) showed that the initial pH had an influence on the autolysis phase.  
519 The cell lysis percentage decreased with increasing initial pH values. The authors obtained the  
520 best toxin titer (67.5 Lf/mL) at an initial pH of 6.1, corresponding to a final pH of 7.75  
521 (Demain and Fang, 2003).

## 522 **6. Nutritional needs of *Clostridium tetani* for bacterial growth and** 523 **toxin production**

### 524 **6.1. Histidine peptide consumption from complex amino acid sources**

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

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

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

548 enzyme was only synthesized in media containing histidine peptides, leading to enhanced  
549 toxin production. Moreover, Fe<sup>2+</sup> stimulated peptidase activity. The histidine peptidase was  
550 able to hydrolyze glycyl-L-histidine and L- $\alpha$ -amino-n-butryl-L-histidine. The hydrolysis of  
551 acetyl-histidine was weaker and needed a high concentration of this peptide, corresponding to  
552 the observation already made by Mueller and Miller (Mueller and Miller, 1956). The  
553 peptidase activity was the same in cell-free assays. Therefore, the superiority of glycyl-  
554 histidine as a peptidase substrate or toxin synthesis effector was not dependent on its  
555 membrane transport. The authors demonstrated that toxin production was directly  
556 proportional to histidine peptide hydrolysis. They also checked that the toxin and the histidine  
557 peptidase were not the same protein (Miller et al., 1960). Later, Helting et al. found this  
558 enzyme in the supernatant and confirmed that it was active toward glycyl-histidine and not  
559 responsible for the proteolysis to a mature toxin (Helting et al., 1979).

## 560 **6.2. Casein peptide consumption from complex amino acid sources**

561 To further evaluate the impact of the casein pancreatic digest (NZ-Case) and identify the  
562 casein peptides with an effect on toxin synthesis, cultivation was performed for NZ-Case  
563 fractions with a Harvard strain in modified Massachusetts medium (supplemented with 1.01  
564 g/L KH<sub>2</sub>PO<sub>4</sub>, 0.72 g/L CaCl<sub>2</sub> and 0.36 g/L active charcoal). Porfirio et al. (1997) found that  
565 only the omission of the neutral fraction of the casein digest decreased toxin production, by  
566 approximately 30 %, suggesting that this fraction contains one or several elements promoting  
567 toxin synthesis. The omission of the acidic or the basic fraction of the casein digest did not  
568 affect the toxin titer. Hence, they purified peptides from the neutral part and individually  
569 added them to the Massachusetts medium. They showed that the following peptides were the  
570 most active in toxin production, with a final toxin titer reaching 200 % to 265 % of the toxin  
571 production in Massachusetts medium:

- 572 - Ile – Pro – Ile – Gln – Tyr – Val
- 573 - Val – Leu – Gly – Pro – Val
- 574 - Ala – Val – Pro – Tyr – Pro – Gln
- 575 - Asp – Met – Pro – Ile
- 576 - Val – Ala – Pro – Phe – Pro – Glu – Val – Phe
- 577 - Glu – Met – Pro – Phe – Pro – Lys

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

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

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

592 - Val – Pro – Gln – Leu – Glu – Ile – Val

593 - Val – Tyr – Pro – Phe – Pro – Gly – Pro – Ile

594 The last peptide harbors the Pro-Phe-Pro pattern, which was described by Porfirio *et al.*  
595 (1997) as a zinc protease inhibitor.

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

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

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

### 606 **6.3. Free amino acid consumption for *C. tetani* growth and toxin production**

607 Despite the influence of peptides in toxin production, studies were conducted to design a  
608 chemically defined medium that could support *C. tetani* growth and toxin production. Two  
609 different media were evaluated (Table 4-A) (Feeney et al., 1943b; Licona-Cassani et al.,  
610 2016)). Glucose was present at the same concentration in both media, but the initial

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

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

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

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

<b>Tryptophan</b>	0.05 (L-)	0.1 (L-)
<b>Tyrosine</b>	0.3 (L-)	0.1 (L-)
<b>Valine</b>	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 <i>et al.</i> , 1943	Yu <i>et al.</i> , 2009	Licona-Cassani <i>et al.</i> , 2016	
	Harvard strain On chemically defined medium (Table 4-A)	E88 strain <sup>1</sup> Deficient in amino acid biosynthetic pathways	E88 strain <u>Approximate consumption</u>	
			On modified Massachusetts medium <sup>2</sup>	On chemically defined medium (Table 4-A)
<b>Arginine</b>	essential	-	0 %	20 %
<b>Asparagine</b>	not experimented	-	90 %	100 %
<b>Aspartate</b>	less effective	-	100 %	100 %
<b>Cysteine</b>	not experimented	-	90 %	not analyzed
<b>Glutamate</b>	stimulated	-	> 95 %	100 %
<b>Glutamine</b>	not experimented	-	90 %	100 %
<b>Histidine</b>	essential	auxotrophic	> 95 %	100 %
<b>Isoleucine</b>	essential	auxotrophic	0 %	15 %
<b>Leucine</b>	essential	auxotrophic	0 %	60 %
<b>Lysine</b>	less effective	auxotrophic	0 %	25 %
<b>Methionine</b>	less effective	auxotrophic	75 %	100 %
<b>Phenylalanine</b>	less effective	auxotrophic	0 %	20 %
<b>Serine</b>	stimulated	-	> 95 %	100 %
<b>Threonine</b>	stimulated	-	> 95 %	100 %
<b>Tryptophan</b>	essential <sup>3</sup>	auxotrophic	0 %	0 %
<b>Tyrosine</b>	essential	-	100 %	60 %
<b>Valine</b>	essential	auxotrophic	0 %	0 %
<b>Growth</b>	X <sub>max</sub> = 80-85 % of the X <sub>max</sub> obtained in Mueller and Miller medium	-	μ = 0.24 h <sup>-1</sup> during exponential growth, then μ = 0.083 h <sup>-1</sup> during slower growth	μ = 0.69 h <sup>-1</sup>

629 <sup>1</sup> Strain not specified; probably E88, as that was the only one sequenced at that time630 <sup>2</sup> Massachusetts medium supplemented with cystine, uracil and vitamins and with trace  
631 amounts of FeCl<sub>3</sub>

632 <sup>3</sup> Tryptophan was not tested in this chemically defined medium but was identified as being  
633 essential in a previous experiment in complex medium (Mueller & Miller, 1942).

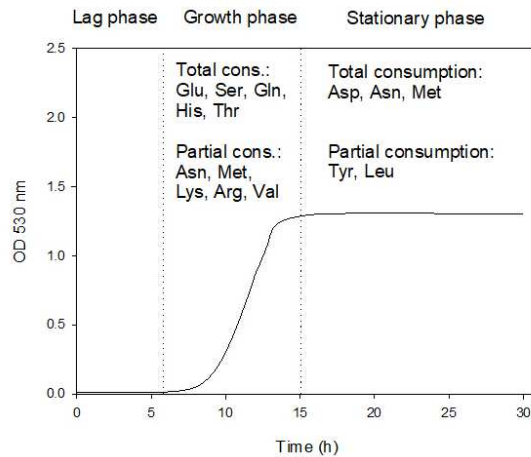
634 Essential: no growth when the amino acid was omitted. Stimulated: little growth after 48 h of  
635 culture when the amino acid was omitted. Less effective: Good growth after 48 h of culture  
636 when the amino acid was omitted.

637  $X_{max}$ : maximum biomass concentration;  $\mu$ : specific growth rate

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

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

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



660

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

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

686 supplementation could result in amino acid overflow, leading to the accumulation of  
687 metabolic intermediates or by-products, which could inhibit *tetR* transcription (Orellana et al.,  
688 2020). The studies presented in Table 4-B do not provide information on the effect of cysteine  
689 on the *C. tetani* growth requirements. In complex medium, addition of cystine (Cys-Cys  
690 peptide) after autoclaving instead of before autoclaving decreased toxin production, probably  
691 because of the effect of heat on cystine. Moreover, cystine inhibited toxin synthesis when  
692 added at a concentration of more than 125 mg/L (Latham et al., 1962).

#### 693 **6.4. Glucose consumption during *C. tetani* cultivation**

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

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

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

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



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

725 Toxin (Lf/mL)

$$726 \quad = 46.29 + 5.55 \left( \frac{G_0}{4} - 2 \right) + 26.82 \left( \frac{NZ_0}{12.5} - 2 \right) - 6.39 \left( \frac{G_0}{4} - 2 \right)^2 - 9.04 \left( \frac{NZ_0}{12.5} \right. \\ 727 \quad \left. - 2 \right)^2$$

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

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

## 731 **6.5. Effects of inorganic compounds on *C. tetani* growth and toxin production**

### 732 **6.5.1 Iron**

733 The role of some growth factors can depend on other medium components. Lerner et al.  
734 (1949) related iron concentration to glucose consumption in Mueller and Miller medium.  
735 Cells in iron-deficient medium were unable to ferment glucose. Glucose consumption was  
736 semilogarithmically proportional to iron content and was estimated through carbon dioxide  
737 production. They suggested that an enzyme or coenzyme of the glucose pathway was iron  
738 dependent. Later, this enzyme was identified as pyruvate:ferredoxin oxidoreductase,  
739 containing Fe-S clusters, which catalyzes pyruvate degradation to acetyl-CoA (Bruggemann  
740 and Gottschalk, 2004). This enzyme needs oxidized ferredoxin as a substrate and releases it in  
741 its reduced form. Lerner *et al.* (1949) also experimented with glutamine addition in iron-  
742 deficient complex medium. This resulted in stimulated carbon dioxide production,  
743 approaching the production levels observed in high-iron medium. Thus, addition of glutamine  
744 could overcome iron deficiency (Lerner and Mueller, 1949).

745 According to Feeney et al. (1943), on casein hydrolysate medium, the optimum iron  
746 concentration was 50 mg/L reduced iron (powder) (Feeney et al., 1943a) or 0.3 mg/L  $\text{FeSO}_4^-$

747 .7H<sub>2</sub>O (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<sup>2+</sup> and Fe<sup>3+</sup>)  
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<sub>3</sub>), even though  
771 it was diluted 1000x compared to the level in the initial Massachusetts medium (32 mg/L of  
772 FeCl<sub>3</sub>.6 H<sub>2</sub>O). They determined that the total intracellular iron (Fe<sup>2+</sup> and Fe<sup>3+</sup>) 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<sup>2+</sup> transport genes (CTC451-452,  
776 CTC534) were expressed in both growth phases, whereas Fe<sup>3+</sup> 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

780                   **6.5.2 Mineral salts**

781   Mueller et al., 1943 reported that potassium, magnesium and phosphate inorganic salts were  
782   required for *C. tetani* growth (Mueller et al., 1943). In continuous culture, Zacharias and  
783   Björklund (1968) showed that the addition of 0.1 g/L potassium chloride in modified  
784   Massachusetts medium doubled the production of toxin (70 Lf/mL to 130 Lf/mL) in a  
785   Harvard strain derivative (strain 107). They pointed out that potassium has the property of  
786   increasing the permeability of the cell membrane. They observed the opposite phenomenon  
787   (70 Lf/mL to 40 LF/mL) with the addition of 0.15 g/L calcium chloride, which is known to  
788   reduce cell membrane permeability (Zacharias and Bjorklund, 1968). In TGY flask cultures of  
789   strain CN655, the optimal inorganic phosphate concentration for toxin production was found  
790   to be 40 mM (two-fold the extracellular toxin concentration in comparison to TGY without  
791   phosphate addition). Phosphate supplementation increased the transcription of *tetX* but not  
792   that of its transcriptional regulator *tetR*. Thus, the authors suggested that toxin regulation by  
793   phosphate is not related to TetR (Chapeton-Montes et al., 2020).

794                   **6.5.3 Vitamins**

795   Vitamins and trace elements used to be supplied by beef heart infusion into Mueller and  
796   Miller medium for *C. tetani* growth. Latham et al. (1962) showed that beef heart infusion was  
797   not essential for growth and toxin production. Beef heart infusion can successfully be  
798   substituted with nicotinic acid, vitamin B12 and iron (Latham et al., 1962). When Hy-Soy®  
799   peptone was used, vitamin addition was not required. Hy-Soy® peptone may contain enough  
800   uracil, calcium pantothenate, thiamine, riboflavin, pyridoxal and biotin for *C. tetani* growth  
801   and toxin production. This complex material also satisfied the tyrosine and cystine  
802   requirements, as supplementation of these materials was no longer required (Demain et al.,  
803   2007).

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

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

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

## 814 **7. Conclusion**

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

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

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## 846 **Conflicts of interest**

847 The authors declare no conflicts of interest.

848

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850

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