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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**

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

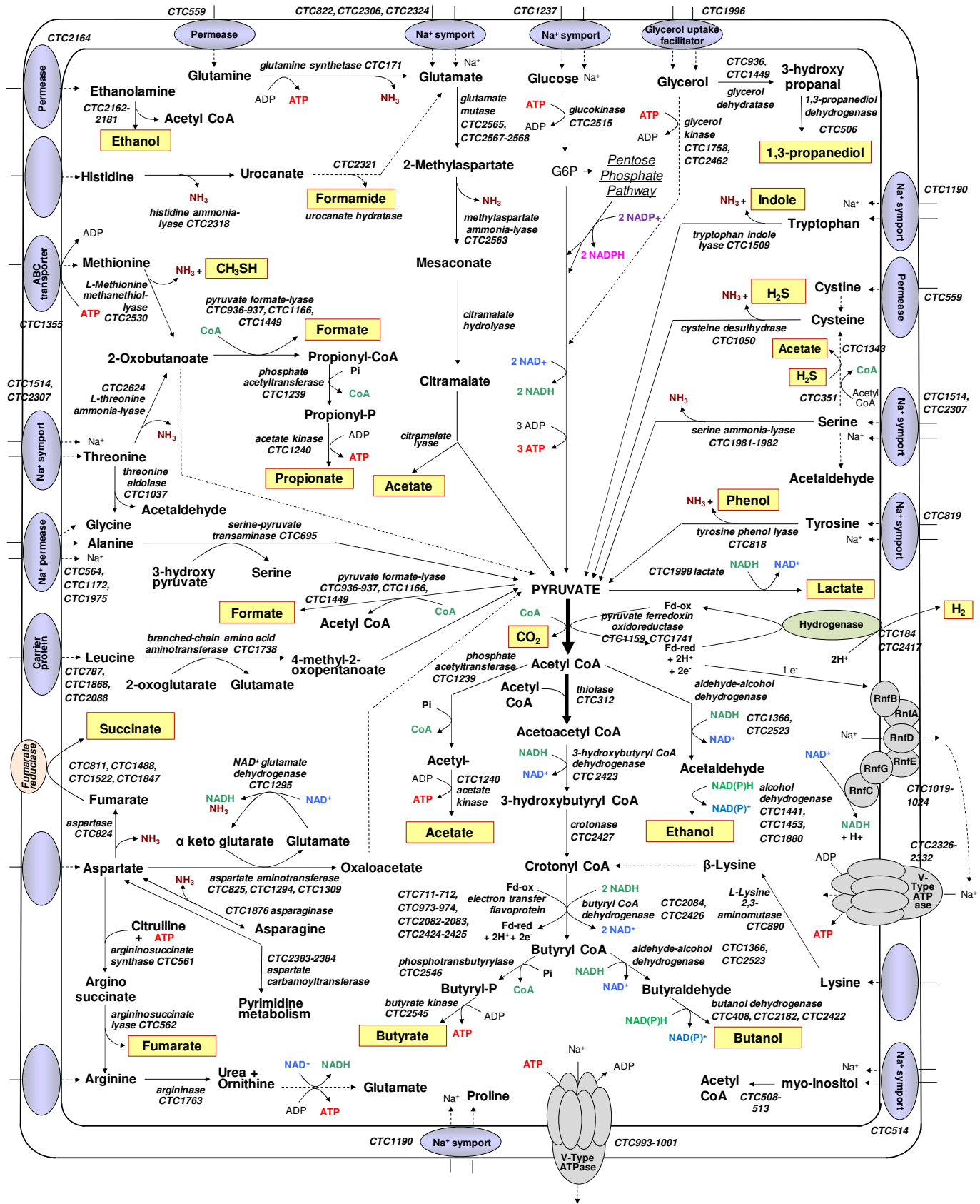
Amino acid	Major degradation pathways	Minor degradation pathways
Aspartate	Glutamate + Oxaloacetate	Ammonium + Fumarate
Glutamate	Acetate + Butyrate + Pyruvate + Ammonium	2-oxoglutarate + Ammonium n-Formimino-L-Glutamate
Histidine	Glutamate	Histamine
Methionine	2-Oxobutanoate + Propionyl-CoA Propionate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

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 **Figure 1: *Clostridium tetani* central metabolism**

240 *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⁺ flow, which can be converted to
257 dihydrogen by hydrogenases or used by ferredoxin NAD⁺ 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⁺, 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⁺ 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 F₀-F₁-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.

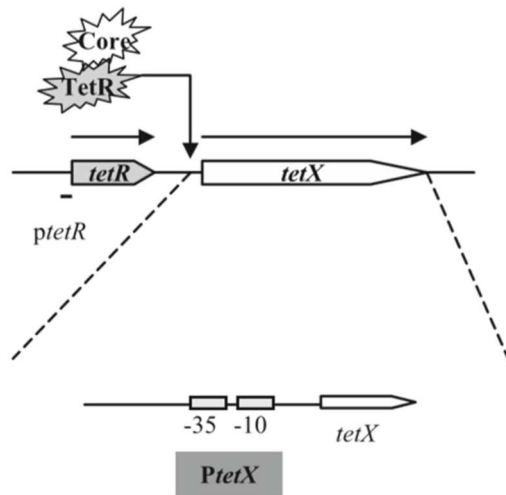
278 An H⁺ pump (V-Type pyrophosphatase CTC383), coupled with the cleavage of inorganic
279 pyrophosphate, was also found in *C. tetani*, which is uncommon in bacteria. In addition, a
280 specific sodium ABC transporter (CTC1485) and several H⁺/Na⁺ antiporters (CTC567,
281 CTC901, CTC1183, CTC1423, CTC1853, 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 σ^{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 Chapeton-Montes et al. (2020) showed that toxin synthesis was also regulated by CodY. This
320 protein, often involved in toxin and virulence regulation in other Gram-positive pathogenic
321 bacteria, exhibited binding to the *tetX* promoter. This increased *tetX* transcription, which
322 positively regulated toxin synthesis. As CodY is known to respond to starvation conditions,
323 the authors suggested that this toxin regulation could be related to nutritional stress
324 (Chapeton-Montes et al., 2020). Comparative transcriptional analysis of E88 grown in
325 complex medium supplemented or not with an amino acid mixture suggested also that toxin
326 production and virulence factors were related (Orellana et al., 2020). Specifically, the
327 decrease in expression of virulence-related surface/cell-adhesion genes (CTC769-770,
328 CTC772, CTC776-777) and flagellar genes considered as virulence-related genes in other
329 pathogens (CTC1653-1679) affected toxin production. However, the genes encoding the
330 homologous TCS of the virulence genes VirS/VirR in *C. perfringens* showed no difference in
331 expression, regardless of the tetanus toxin production level.
332

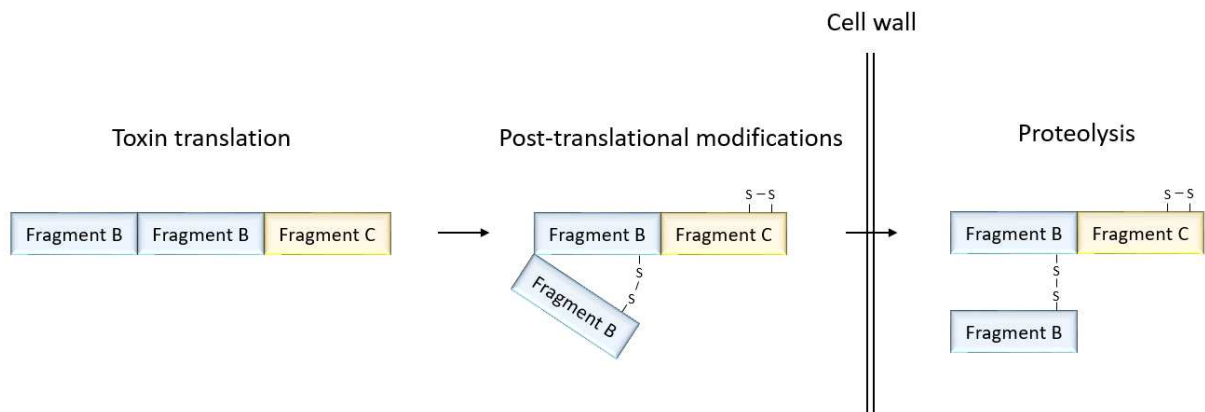
333 *C. tetani* phenotypes seemed to depend on toxin synthesis, with the long-chained filaments
334 appearing in the culture with the highest toxin production (Orellana et al., 2020). Yet, the
335 expression of cell division and cell wall elongation genes was not linked to toxin synthesis
336 when the 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).

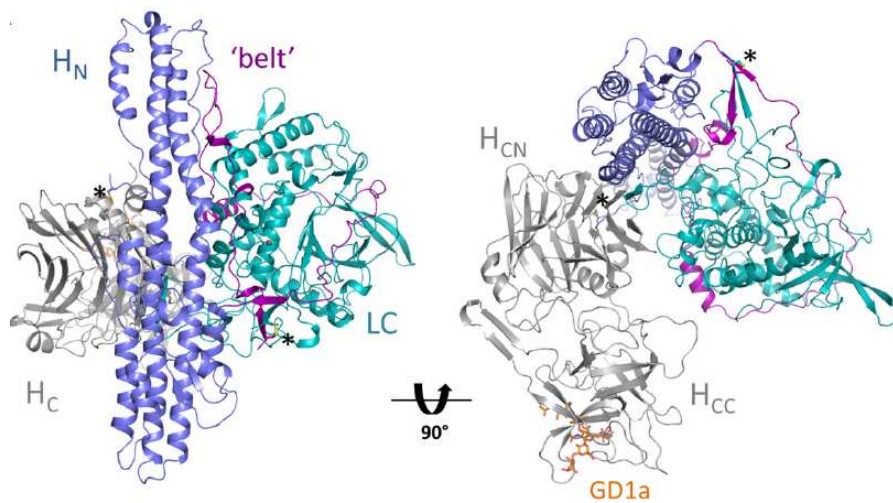
365 Schiavo et al. (1992) identified one zinc atom linked to the light chain playing a catalytic role.
366 This atom is linked to a two-histidine motif, characteristic of metalloendopeptidases. Without
367 zinc, the tetanus toxin cannot inhibit neurotransmitters and therefore cannot cause disease
368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

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	-
Na₂HPO₄	2 g	-	0.5 g	-
KH₂PO₄	0.15 g	-	0.175 g	-
MgSO₄	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 B₁₂	-	0.05 µg	-	-
Reduced iron (powder)	0.5 g	-	0.5 g	-
FeCl₃ · 6 H₂O	-	32 mg	-	-
pH adjustment (before autoclave)	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

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	μ = 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
(Licon-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

456 Abbreviation: Lf/mL, limit of flocculation per milliliter (tetanus toxin unit); μ , 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 ± 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 h^{-1} to 0.169 h^{-1}), ii) temperatures (32°C to 39°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⁻¹, 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⁻¹ to 0.125 h⁻¹ 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- α -amino-n-butyryl-L-histidine; ii) β -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, Fe²⁺ stimulated peptidase activity. The histidine peptidase was
550 able to hydrolyze glycyl-L-histidine and L- α -amino-n-butyryl-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 KH₂PO₄, 0.72 g/L CaCl₂ 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	
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 <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 ¹ Deficient in amino acid biosynthetic pathways	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	-	μ = 0.24 h ⁻¹ during exponential growth, then μ = 0.083 h ⁻¹ during slower growth	μ = 0.69 h ⁻¹

629 ¹ Strain not specified; probably E88, as that was the only one sequenced at that time630 ² Massachusetts medium supplemented with cystine, uracil and vitamins and with trace
631 amounts of FeCl₃

632 ³ 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).

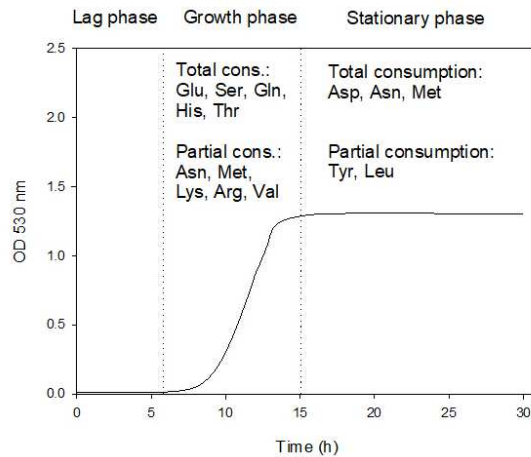
634 Essential: no growth when the amino acid was omitted. Stimulated: little growth after 48 h of
635 culture when the amino acid was omitted. Less effective: Good growth after 48 h of culture
636 when the amino acid was omitted.

637 X_{max} : maximum biomass concentration; μ : 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⁻¹) 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⁻¹ instead of 0.06 h⁻¹ 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 FeSO_4^-

747 .7H₂O (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 (Fe²⁺ and Fe³⁺)
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 FeCl₃), even though
771 it was diluted 1000x compared to the level in the initial Massachusetts medium (32 mg/L of
772 FeCl₃.6 H₂O). They determined that the total intracellular iron (Fe²⁺ and Fe³⁺) 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 Fe²⁺ transport genes (CTC451-452,
776 CTC534) were expressed in both growth phases, whereas Fe³⁺ 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 Licon-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 (Licon-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

849 **References**

850

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