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Clostridioides difficile peptidoglycan modifications

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10 Abstract

11 The cortex and peptidoglycan of *Clostridioides difficile* have been poorly investigated. This last decade,
12 the interest increased because these two structures are highly modified and these modifications may
13 be involved in antimicrobial resistance. For example, *C. difficile* peptidoglycan deacetylation was
14 recently reported to be involved in lysozyme resistance. Modifications may also be important for spore
15 cortex synthesis or spore germination, which is essential in *C. difficile* pathogenesis. As such, the
16 enzymes responsible for modifications of the peptidoglycan and/or cortex could be new drug target
17 candidates or used as anti-*C. difficile* agents, as seen for the CD11 autolysin. In this review, we focused
18 on *C. difficile* peptidoglycan and cortex and compared their structures with those of other well studied
19 bacteria.

20

21 Introduction

22 Peptidoglycan is specific and unique to bacteria [1]. It surrounds bacteria and is necessary for bacterial
23 growth. In *C. difficile*, this structure is found as a part of the vegetative cell wall but also in the cortex,
24 which is a peptidoglycan-like structure found in the spore [2]. Many peptidoglycan modifications are
25 reported and some may be crucial for the bacterial survival and way of life [3]. In this review, we will
26 focus on the *C. difficile* peptidoglycan and cortex structures and their modifications in comparison with
27 those of other well-studied bacteria.

28

29 Peptidoglycan structure

30 The amino acids found in the pentapeptide stem of *C. difficile* peptidoglycan are similar to that of gram
31 negative or *Bacillus* genus bacteria, including D-Alanine, L-Alanine, D-Glutamate and meso
32 diaminopimelic acid (A₂pm) [4] (Figure 1A). In addition to these amino acids, several new amino acids
33 such as phenylalanine, lysine, valine or modified alanine were identified in low amounts in the
34 mucopeptides of the 630Δ*erm* strain using LC–high-resolution mass spectrometry [5]. Aside from the
35 composition of the pentapeptide stem, the bacterial peptidoglycan of *C. difficile* has several highly
36 unique characteristics. Amongst these characteristics, it contains a majority of A₂pm³→ A₂pm³ cross-
37 links (2,6 diaminopimelic acid linked in position 3 of the peptide of the peptidoglycan) instead of D-
38 Ala→ A₂pm³ cross-links found in the majority of bacteria (Figure 1A) [6]. Through a combination of
39 genetic knock-out studies and characterization of recombinant proteins, three proteins have been
40 shown to be L,D-transpeptidases with partially redundant functions leading to the formation of
41 A₂pm³→ A₂pm³ cross-links [7]. However, discrepancies between these proteins could hint at
42 differences in localization, substrate specificity, antibiotic sensitivity or even essentiality. Indeed,
43 inactivation of *ldt_{cd1}* and *ldt_{cd2}* lead to a decrease in the abundance of A₂pm³→ A₂pm³ cross-links in
44 peptidoglycan. In contrast, no mutant could be generated for *ldt_{cd3}*, suggesting that *ldt_{cd3}* is essential
45 [7]. Recombinant proteins of *Ldt_{cd1}* and *Ldt_{cd2}* were found to be the targets of carbapenems, while this
46 was not seen for the recombinant protein of *Ldt_{cd3}* [7]. Incubation of recombinant proteins with
47 disaccharide-tetrapeptide revealed that *Ldt_{cd2}* and *Ldt_{cd3}* display L,D-transpeptidase and L,D-
48 carboxypeptidase activities. In contrast, the *Ldt_{cd1}* recombinant protein displayed only L,D
49 carboxypeptidase activity in these experimental conditions [7]. Finally, it is important to note that the
50 L,D-transpeptidase encoded by *ldt_{cd2}*, Cpw22, has recently been studied and shown to be involved in
51 cell wall integrity and viability in the R20291 strain [8].

52

53 Peptidoglycan deacetylation

54 The second particularity of the *C. difficile* peptidoglycan is the high *N*-deacetylation of the glucosamine
55 (93 to 97.5% according to the studies) [5,9,10]. Among 12 potential *N*-deacetylases encoded in the
56 genome of *C. difficile*, at least two are involved in the peptidoglycan *N*-deacetylation and lysozyme
57 resistance. These enzymes, PdaV and PgdA, act in synergy and are regulated by the extracytoplasmic
58 function σ^V (sigma factor V) [5,9,10]. *pdaV* expression is fully dependent on σ^V whereas *pgdA* is still
59 expressed in a *csfV* mutant (*csfV* encodes σ^V) suggesting a difference of regulation for both genes. Each
60 individual mutant, *pgdA* or *csfV*, is slightly more sensitive to lysozyme than the parental strain (4-fold
61 in the 630Δ*erm* and 2-fold and 8-fold respectively in the R20291 [5,10]). In contrast, the double mutant
62 is more than 130-fold more sensitive to lysozyme in the 630Δ*erm* strain and 1000-fold in the R20291
63 strain [5,9]. In other pathogenic bacteria such as *Streptococcus pneumoniae* or *Listeria*
64 *monocytogenes*, the absence of peptidoglycan *N*-deacetylation altered the virulence [11,12]. In

65 contrast, *in vivo* virulence studies done in *C. difficile* showed that although the intestinal colonization
66 is lower for the double mutant $\Delta pgdA\ csfV$ compared to the parental strain, the double mutant is more
67 virulent than the parental strain [5]. A possible explanation for this observation, that need to be further
68 investigated, is that the increased lysozyme sensitivity of the double mutant, shown *in vitro*, could lead
69 to a stronger bacterial lysis during *in vivo* infection. This could facilitate the release of toxins TcdA and
70 TcdB [13,14], ultimately leading to a higher mortality of the host.

71

72 Peptidoglycan modifications in the presence of vancomycin

73 Peptidoglycan modifications may be also involved in antibiotic resistance [15]. Indeed, bacterial
74 resistance to vancomycin has been linked to the presence of a *van* gene cluster [16]. While 74% of
75 *C. difficile* strains possess a *vanG_{cd}* gene cluster [17,18], only a few clinical strains have been found to
76 harbor a vancomycin resistance [19]. The *vanG_{cd}* gene cluster is composed of two operons: a regulation
77 operon (*vanR* and *vanS*) and a resistance operon (*vanG*, *vanXY* and *vanT*). The regulation operon is
78 constitutively expressed, whereas the resistance operon is induced by the presence of vancomycin
79 through the two-component system VanS-VanR. In the presence of vancomycin, the peptidoglycan
80 precursors are modified and close to 40% of them are UDP-MurNac-pentapeptide[D-Ser] instead of
81 the natural peptidoglycan precursor UDP-MurNac-pentapeptide[D-Ala] [17]. This low rate of modified
82 precursors may lead to the slight change in vancomycin susceptibility between the *vanG* or *vanR*
83 mutants (MIC 0.75 mg L⁻¹) and the parental strain (MIC 1.5 mg L⁻¹). The lack of resistance and low rate
84 of modified precursor UDP-MurNac-pentapeptide[D-Ser] may be explained by the absence of D,D-
85 carboxypeptidase activity of VanXY and the fact that MurF has a preferential use of lipid II ending with
86 D-Ala-D-Ala instead of D-Ala-D-Ser [17]. In addition, in the presence of vancomycin, Ammam *et al.*
87 reported amidation of a subset of peptidoglycan precursors [20]. This modification was only found in
88 the presence of vancomycin and was independent from the VanS-VanR two-component system.
89 Intriguingly, when the asparagine synthetase *asnB* was overexpressed, more than 90% of the
90 peptidoglycan precursors were amidated, but the growth or morphology of the strain was similar to
91 that of the parental strain [20]. In contrast, the overexpression of *asnB* led to a decrease of the
92 vancomycin MIC from 2-3 to 1-1.5 mg L⁻¹ [20]. Finally, Shen *et al.* recently showed that a weak
93 expression of the resistance operon is involved in the lack of vancomycin resistance [19]. Indeed,
94 punctual mutations in *vanS* or *vanR* may increase the expression of the resistance operon, leading to
95 an increase of the MIC from 4 to 16 mg L⁻¹. In addition, Pu *et al.* identified a transferable plasmid (pX18-
96 498) that is found in clinical strains associated with vancomycin treatment failure in CDI patients [21].
97 This plasmid may result in decreased susceptibility to vancomycin. The combination of such a plasmid
98 with mutations in *vanR* and/or *vanS* may result in increasing rates of vancomycin resistance in *C.*
99 *difficile* strains.

100

101 Peptidoglycan autolysins

102 Autolysins are enzymes that maturate the peptidoglycan and are be involved in toxins release [22].
103 Several were recently predicted in *C. difficile* [23]. Among them, the N-acetylglucosaminidase Acd was
104 studied using *B. subtilis* peptidoglycan (highly acetylated on its glucosamine) [24]. It would be worth
105 to know whether this enzyme is also able to act on deacetylated muropeptides since *C. difficile*
106 peptidoglycan is highly deacetylated. A second autolysin, Cwp19, is a lytic transglycosidase that cleaves
107 the peptidoglycan between the N-acetyl muramic acid and the N-acetyl glucosamine [13,25,26].
108 Interestingly, it was shown that the toxins, TcdA and TcdB, were released due to the autolysis induced
109 by Cwp19, in a process that appears media specific [26]. This autolysin is expressed during the active

110 phase of growth (mainly up to 24h of growth), but its activity is detected during the late stationary
111 phase of growth (from 24h). The hypothesis behind this apparent inconsistency is that a secondary
112 polymer like the PSII or the LTA could modulate its autolytic activity. However, CWP proteins, including
113 Cwp19, are suggested to be placed at the top of the PSII [27] and therefore should be resistant to the
114 shielding-like activity proposed. Two N-acetylmuramoyl-L-alanine amidases, CD11 and CDG, are
115 produced by *C. difficile* prophages and were identified as clinical candidates to target *C. difficile*
116 infection [28]. These enzymes may have a role in the peptidoglycan maturation. However, it was later
117 shown that CD11 is able to kill *C. difficile* bacteria in PBS, but is mostly inactivated by the wall teichoic
118 acids [28]. CwIA is a peptidoglycan hydrolase described in the 630 Δ *erm* (CD630_11350) and in the
119 R20291 (Cwl0971) strains [29,30]. This endopeptidase has three SH3_3 domains (also named SH3_b
120 [31]) and a NLPC/60 super family catalytic domain. CwIA cleaves the stem peptide of the peptidoglycan
121 between the γ -D-Glu and mDAP. CwIA is predominantly found at the septum, and in its absence, *C.*
122 *difficile* has a cell separation defect. In the R20291 strain, *cwIA* was found to be mainly expressed in
123 the exponential phase of growth and in a lesser extent during the stationary phase. Recent work has
124 shown that CwIA is in fact controlled by the Serine/Threonine kinases PrkC and CD630_21480 [29,32].
125 The proposed model is that PrkC is able to phosphorylate CwIA, which leads to cytoplasmic localization
126 of CwIA. In contrast, unphosphorylated CwIA is exported in the cell wall where it is able to cleave the
127 peptidoglycan [29]. This is a particular regulation that was not reported previously.

128

129 Spore cortex

130 The composition and structure of *C. difficile* spore cortex has recently been extensively characterized
131 [33] (Figure 1B). It consists in muropeptides that are predominantly found as monomers
132 (approximately 90% of muropeptides) and a minority found as dimers, leading to a very low cross-
133 linking index (< 5%). Muramic- δ -lactams were detected on 24% of the muropeptides, in contrast with
134 *B. subtilis* [34] or *C. perfringens* [35] where 50% of muropeptides contained muramic- δ -lactams. Unlike
135 species such as *B. subtilis* where a single PdaA N-deacetylase is involved in muramic- δ -lactam synthesis
136 [36], two N-deacetylases were found to be involved in the muramic- δ -lactam synthesis in *C. difficile*:
137 PdaA1 and PdaA2 (CD630_14300 and CD630_27190 respectively) [33]. These two enzymes are forming
138 the δ -lactams after the processing of muramic acid and its stem peptide by GerS and CwID [37,38]. A
139 mutant of *pdaA1* and *pdaA2* exhibits a decreased sporulation, a decreased heat resistance, delayed
140 virulence in a hamster infection model, and an altered germination [33]. In contrast, *B. subtilis* cannot
141 germinate if the muropeptides lack muramic- δ -lactams [39]. Interestingly, enzymes such as the cortex
142 lytic enzyme SleC are sensitive to the presence of δ -lactams (see *C. difficile* spore germination section)
143 [40-42]. Another distinctive characteristic of the *C. difficile* cortex is that 22% of the muropeptides in
144 the cortex were not substituted, in contrast with *B. subtilis* where most of them were substituted with
145 L-alanine. Finally, 55% to 61% of the muropeptides were N-deacetylated in the cortex of *C. difficile*
146 630 Δ *erm* whereas no or very low N-deacetylation was observed in the cortex of *B. subtilis* or
147 *C. perfringens* (10%) [33-35]. This high percentage of N-deacetylation in the cortex of *C. difficile* may
148 be driven by the high number of N-deacetylases. One of the enzymes potentially involved in the spore
149 cortex N-deacetylation is PgdB (CD630_32570) [33]. Indeed, *pgdB* was poorly expressed during the
150 bacterial growth and is under control of σ^E factor [33,43]. Moreover, a slight decrease in glucosamine
151 N-deacetylation (5%) of the spore cortex was observed in the Δ *pgdB* mutant [5]. This suggest that other
152 N-deacetylases are involved in the N-deacetylation of the spore cortex in *C. difficile*. Spore cortex is
153 synthesized by both the mother cell and the forespore [2,44]. During the engulfment stage of
154 sporulation, the bacterial peptidoglycan is separated from the spore cortex by specific autolysins such
155 as SpoIID and SpoIIP [37,45,46] (see *C. difficile* spore assembly section), and this process relies on the
156 structural differences between bacterial peptidoglycan and spore cortex. Since very little is known

157 about N-deacetylation in the spore cortex, the impact of this modification has to be further
158 investigated to fully understand its contribution to the sporulation and germination processes.

159

160 Conclusions

161 Some advances have been highlighted in studying the peptidoglycan and cortex of *C. difficile* in the last
162 decade. A deeper study of the peptidoglycan structure, biogenesis, recycling and degradation will allow
163 a better understanding of the role of the *C. difficile* peptidoglycan in the toxin release and in the
164 antibiotic and antimicrobial resistances. Finally, the recent analysis of the cortex structure will allow a
165 better understanding of the germination process through its hydrolysis.

166

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169

170 Declaration of interest

171 None

172

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174 * Of special interest

175 ** Of outstanding interest

176

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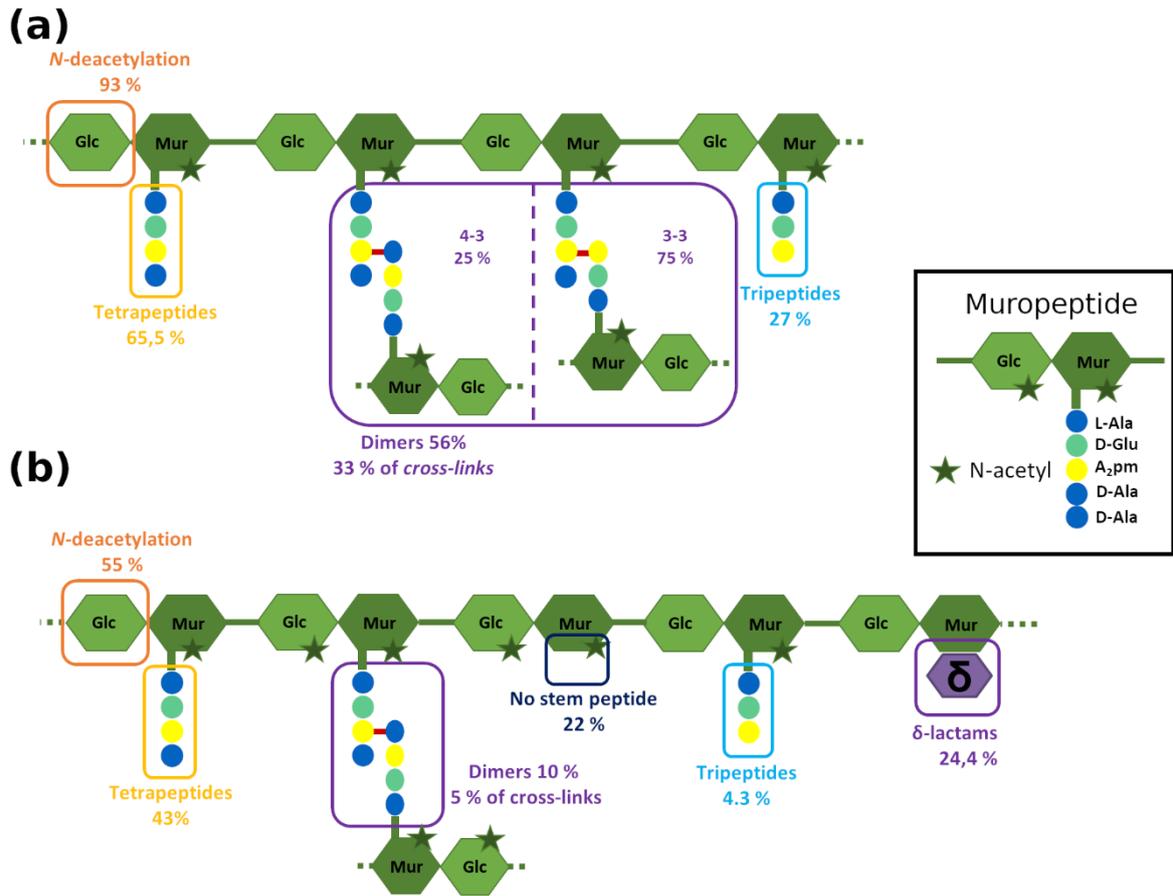
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325 Figure 1: Peptidoglycan and cortex structures

326 Peptidoglycan (A) and cortex (B) structures of *C. difficile* are presented. A minimal structure named
 327 muropeptide is represented in the right-hand side framed section of the figure. It is composed of two
 328 sugars, a muramic acid and a glucosamine, and a peptide which contains L-alanine (L-Ala), D-glutamate
 329 (D-Glu), 2,6 diaminopimelic acid (A₂pm) and D-Alanine (D-Ala) as represented. Peptidoglycan and
 330 cortex structures are represented here by assembling of typical muropeptides linked together, forming
 331 a chain of glycan. The principal modifications are indicated and highlighted by a colored box.

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