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Abstract

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

Introduction

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

Peptidoglycan structure

The amino acids found in the pentapeptide stem of *C. difficile* peptidoglycan are similar to that of gram negative or *Bacillus* genus bacteria, including D-Alanine, L-Alanine, D-Glutamate and meso diaminopimelic acid (A_2pm) [4] (Figure 1A). In addition to these amino acids, several new amino acids such as phenylalanine, lysine, valine or modified alanine were identified in low amounts in the muropeptidesof the 630Δ*erm* strain using LC–high-resolution mass spectrometry [5]. Aside from the composition of the pentapeptide stem, the bacterial peptidoglycan of *C. difficile* has several highly unique characteristics. Amongst these characteristics, it contains a majority of $A_2pm^3 \rightarrow A_2pm^3$ cross-links (2,6 diaminopimelic acid linked in position 3 of the peptide of the peptidoglycan) instead of D-Ala$\rightarrow A_2pm^3$ cross-links found in the majority of bacteria (Figure 1A) [6]. Through a combination of genetic knock-out studies and characterization of recombinant proteins, three proteins have been shown to be L,D-transpeptidases with partially redundant functions leading to the formation of $A_2pm^3 \rightarrow A_2pm^3$ cross-links [7]. However, discrepancies between these proteins could hint at differences in localization, substrate specificity, antibiotic sensitivity or even essentiality. Indeed, inactivation of *ldt*$_{cd1}$ and *ldt*$_{cd2}$ lead to a decrease in the abundance of $A_2pm^3 \rightarrow A_2pm^3$ cross-links in peptidoglycan. In contrast, no mutant could be generated for *ldt*$_{cd3}$, suggesting that *ldt*$_{cd3}$ is essential [7]. Recombinant proteins of Ldt$_{cd1}$ and Ldt$_{cd2}$ were found to be the targets of carbapenems, while this was not seen for the recombinant protein of Ldt$_{cd3}$ [7]. Incubation of recombinant proteins with disaccharide-tetrapeptide revealed that Ldt$_{cd2}$ and Ldt$_{cd3}$ display L,D-transpeptidase and L,D-carboxypeptidase activities. In contrast, the Ldt$_{cd1}$ recombinant protein displayed only L,D carboxypeptidase activity in these experimental conditions [7]. Finally, it is important to note that the L,D-transpeptidase encoded by *ldt*$_{cd2}$, Cpw22, has recently been studied and shown to be involved in cell wall integrity and viability in the R20291 strain [8].

Peptidoglycan deacetylation

The second particularity of the *C. difficile* peptidoglycan is the high *N*-deacetylation of the glucosamine (93 to 97.5% according to the studies) [5,9,10]. Among 12 potential *N*-deacetylases encoded in the genome of *C. difficile*, at least two are involved in the peptidoglycan *N*-deacetylation and lysozyme resistance. These enzymes, PdaV and PgdA, act in synergy and are regulated by the extracytoplasmic function σ^V (sigma factor V) [5,9,10]. *pdaV* expression is fully dependent on σ^V whereas *pgdA* is still expressed in a *csfV* mutant (*csfV* encodes σ^V) suggesting a difference of regulation for both genes. Each individual mutant, *pgdA* or *csfV*, is slightly more sensitive to lysozyme than the parental strain (4-fold in the 630Δ*erm* and 2-fold and 8-fold respectively in the R20291 [5,10]). In contrast, the double mutant is more than 130-fold more sensitive to lysozyme in the 630Δ*erm* strain and 1000-fold in the R20291 strain [5,9]. In other pathogenic bacteria such as *Streptococcus pneumoniae* or *Listeria monocytogenes*, the absence of peptidoglycan *N*-deacetylation altered the virulence [11,12]. In contrast, *in vivo* virulence studies done in *C. difficile* showed that although the intestinal colonization is lower for the double mutant *ΔpgdA csfV* compared to the parental strain, the double mutant is more virulent than the parental strain [5]. A possible explanation for this observation, that need to be further investigated, is that the increased lysozyme sensitivity of the double mutant, shown *in vitro*, could lead to a stronger bacterial lysis during *in vivo* infection. This could facilitate the release of toxins TcdA and TcdB [13,14], ultimately leading to a higher mortality of the host.

Peptidoglycan modifications in the presence of vancomycin

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

Peptidoglycan autolysins

Autolysins are enzymes that maturate the peptidoglycan and are be involved in toxins release [22]. Several were recently predicted in *C. difficile* [23]. Among them, the *N*-acetylglucosaminidase Acd was studied using *B. subtilis* peptidoglycan (highly acetylated on its glucosamine) [24]. It would be worth to know whether this enzyme is also able to act on deacetylated muropeptides since *C. difficile* peptidoglycan is highly deacetylated. A second autolysin, Cwp19, is a lytic transglycosidase that cleaves the peptidoglycan between the N-acetyl muramic acid and the N-acetyl glucosamine [13,25,26]. Interestingly, it was shown that the toxins, TcdA and TcdB, were released due to the autolysis induced by Cwp19, in a process that appears media specific [26]. This autolysin is expressed during the active phase of growth (mainly up to 24h of growth), but its activity is detected during the late stationary phase of growth (from 24h). The hypothesis behind this apparent inconsistency is that a secondary polymer like the PSII or the LTA could modulate its autolytic activity. However, CWP proteins, including Cwp19, are suggested to be placed at the top of the PSII [27] and therefore should be resistant to the shielding-like activity proposed. Two N-acetylmuramoyl-L-alanine amidases, CD11 and CDG, are produced by *C. difficile* prophages and were identified as clinical candidates to target *C. difficile* infection [28]. These enzymes may have a role in the peptidoglycan maturation. However, it was later shown that CD11 is able to kill *C. difficile* bacteria in PBS, but is mostly inactivated by the wall teichoic acids [28]. CwlA is a peptidoglycan hydrolase described in the 630Δ*erm* (CD630_11350) and in the R20291 (Cwl0971) strains [29,30]. This endopeptidase has three SH3_3 domains (also named SH3_b [31]) and a NLPC/60 super family catalytic domain. CwlA cleaves the stem peptide of the peptidoglycan between the γ-D-Glu and mDAP. CwlA is predominantly found at the septum, and in its absence, *C. difficile* has a cell separation defect. In the R20291 strain, *cwlA* was found to be mainly expressed in the exponential phase of growth and in a lesser extent during the stationary phase. Recent work has shown that CwlA is in fact controlled by the Serine/Threonine kinases PrkC and CD630_21480 [29,32]. The proposed model is that PrkC is able to phosphorylate CwlA, which leads to cytoplasmic localization of CwlA. In contrast, unphosphorylated CwlA is exported in the cell wall where it is able to cleave the peptidoglycan [29]. This is a particular regulation that was not reported previously.

Spore cortex

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

Conclusions

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

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Declaration of interest

None

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* Of special interest

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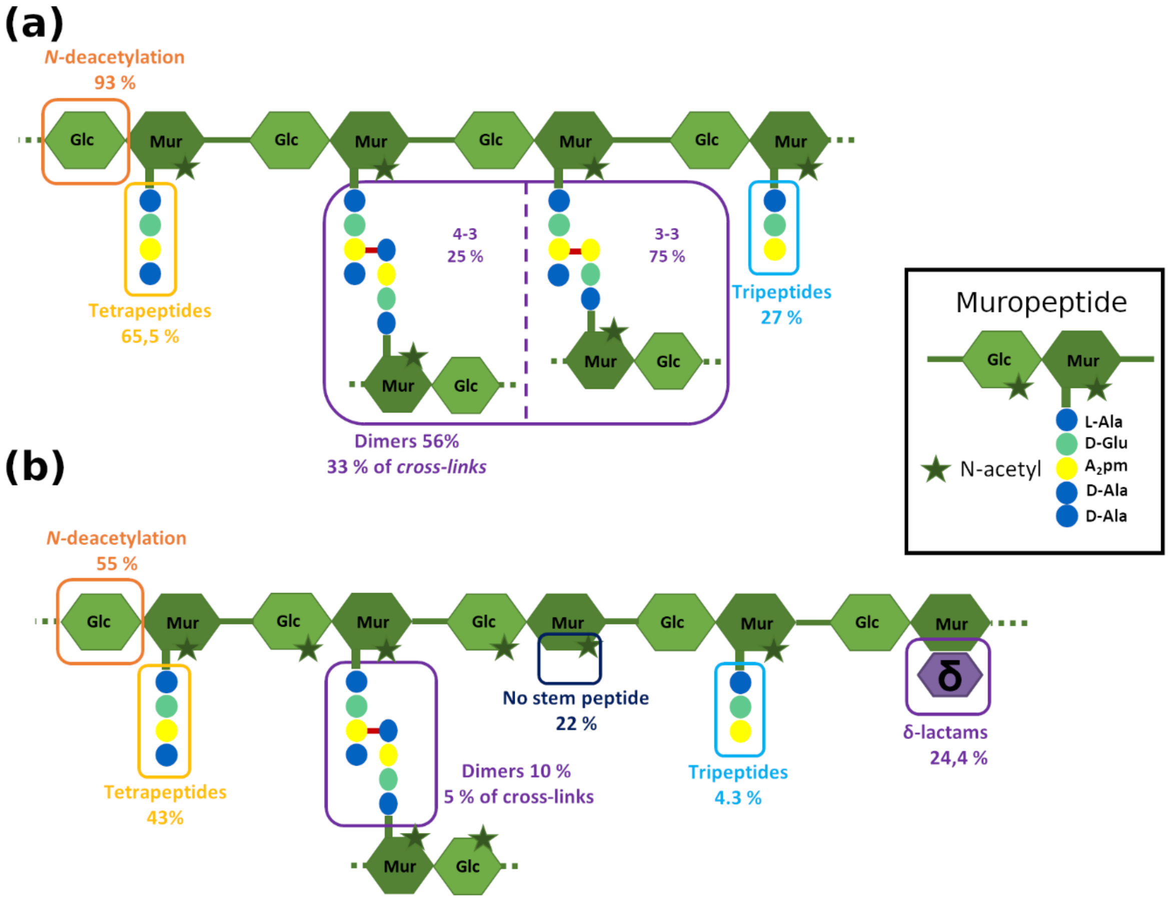

Figure 1: Peptidoglycan and cortex structures

Peptidoglycan (A) and cortex (B) structures of *C. difficile* are presented. A minimal structure named muropeptide is represented in the right-hand side framed section of the figure. It is composed of two sugars, a muramic acid and a glucosamine, and a peptide which contains L-alanine (L-Ala), D-glutamate (D-Glu), 2,6 diaminopimelic acid (A_2pm) and D-Alanine (D-Ala) as represented. Peptidoglycan and cortex structures are represented here by assembling of typical muropeptides linked together, forming a chain of glycan. The principal modifications are indicated and highlighted by a colored box.