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1 **AsnB is responsible for peptidoglycan precursor amidation in**
2 ***Clostridium difficile* in the presence of vancomycin.**

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23

24 **Abstract**

25 *Clostridium difficile* 630 possesses a cryptic but functional gene cluster *vanG_{Cd}* homologous to the
26 *vanG* operon of *Enterococcus faecalis*. Expression of *vanG_{Cd}* in the presence of subinhibitory
27 concentrations of vancomycin is accompanied by peptidoglycan amidation on the *meso*-DAP residue.
28 In this paper, we report the presence of two potential asparagine synthetase genes named *asnB* and
29 *asnB2* in the *C. difficile* genome whose products were potentially involved in this peptidoglycan
30 structure modification. We found that *asnB* expression was only induced when *C. difficile* was grown
31 in the presence of vancomycin, yet independently from the *vanG_{Cd}* resistance and regulation operons.
32 In addition, peptidoglycan precursors were not amidated when *asnB* was inactivated. No change in
33 vancomycin MIC was observed in the *asnB* mutant strain. In contrast, overexpression of *asnB* resulted
34 in the amidation of most of the *C. difficile* peptidoglycan precursors and in a weak increase of
35 vancomycin susceptibility. AsnB activity was confirmed in *E. coli*. In contrast, the expression of the
36 second asparagine synthetase, AsnB2, was not induced in the presence of vancomycin. In summary,
37 our results demonstrate that AsnB is responsible for peptidoglycan amidation of *C. difficile* in the
38 presence of vancomycin.

39 INTRODUCTION

40 Peptidoglycan (PG) is an essential component of the bacterial cell wall with pleiotropic functions,
41 including maintenance of cell shape and integrity, and anchoring of cell wall proteins (CWP). PG
42 structure is largely conserved, consisting in long glycan chains cross-linked by short peptides. The
43 polysaccharide backbone is made of alternating β -1-4 linked *N*-acetylglucosamine (GlcNAc) and *N*-
44 acetylmuramic acid (MurNAc) motives. The short peptide stems, which are bound to the D-lactoyl
45 groups of MurNAc residues and whose composition and structure vary between bacterial species, are
46 subsequently cross-linked together by transpeptidase activities, generating this giant reticulated
47 peptidoglycan polymer (reviewed in [1]). Among the peptide modifications, amidation, either on D-
48 Glu, D-Asp or *meso*-diaminopimelic acid (DAP) residues, was described in different species,
49 including *Lactobacillus plantarum*, *Mycobacterium tuberculosis*, *Corynebacterium glutamicum* and
50 *Staphylococcus aureus*. In *L. plantarum*, *asnB1*, coding for a glutamine-dependent asparagine
51 synthetase, was shown to be responsible for the amidation of PG *meso*-DAP residues [2]. This
52 modification was involved in the control of L,D-carboxypeptidase activity and its absence affected
53 growth and cell morphology. A *C. glutamicum* gene named *ltsA* was reported to encode an enzyme
54 responsible for *meso*-DAP amidation, whose absence resulted in an abnormal cell shape and increased
55 susceptibility to lysozyme and β -lactam antibiotics [3]. *LtsA*, as *AsnB1*, was expected to belong to
56 the family of glutamine-dependent asparagine synthetases (EC 6.3.5.4). More recently, *meso*-DAP
57 amidation of PG was shown to impact the activity of L,D-transpeptidases in *Enterococcus faecium*
58 and *M. tuberculosis* and to be required for growth of these species [4]. The amidotransferase *AsnB*
59 from *Bacillus subtilis* was also shown to be responsible for *meso*-DAP amidation [5]. In *Lactococcus*
60 *lactis*, *asnH* encodes the asparagine synthetase involved in the amidation of PG D-Asp residues [6]
61 and the absence of this modification increased the cell susceptibility to endogenous autolysins,
62 lysozyme and nisin. In *S. aureus* and other species, D-Glu residues of PG are amidated by two
63 enzymes *GatD* and *MurT* working in concert. *S. aureus* cells lacking this PG modification grew
64 poorly and exhibited an increased susceptibility to β -lactam antibiotics and lysozyme [7, 8].

65
66 *Clostridium difficile* is the main agent responsible for post antibiotic diarrhea and pseudomembranous
67 colitis [9]. Vancomycin, metronidazole and fidaxomicin are the recommended antibiotics used for
68 the treatment of *C. difficile* infections [10]. An ortholog gene cluster of *vanG*, encoding vancomycin
69 resistance in enterococci, was reported in most *C. difficile* clinical isolates [11, 12]. This *vanG_{Cd}*
70 cluster is composed of two operons, one made of *vanR_{Cd}* and *vanS_{Cd}* is involved in the regulation of

71 the *vanG_{Cd}* operon, and the second composed of *vanG_{Cd}*, *vanXY_{Cd}* and *vanT_{Cd}* involved in the
72 modification of PG precursors structure. Thus, in the presence of vancomycin, the *vanG_{Cd}-vanXY_{Cd}-*
73 *vanT_{Cd}* operon was specifically expressed through the *vanRS* regulation operon and PG peptide chains
74 ending in D-Ala-D-Ser instead of D-Ala-D-Ala were detected. This modification leads usually to
75 vancomycin resistance, but, surprisingly in *C. difficile*, no resistance to vancomycin was observed.
76 Moreover, amidation of PG and PG precursors *meso*-DAP residues was shown to occur
77 simultaneously with the D-Ala-D-Ser modification of peptides [13]. This modification was
78 determined by MS-MS analyses [13].

79 The aim of this study was to better understand the appearance of the PG amidation of *C. difficile*
80 which only occurs in the presence of vancomycin. In this study, we identified and characterized two
81 genes encoding potential enzymes responsible for the amidation of PG precursors and PG in *C.*
82 *difficile*.

105

106 **METHODS**

107 **Bacterial strains and growth conditions**

108 *Escherichia coli* TG1 and HB101(pRK24) strains were grown at 37°C in LB medium (Luria Broth,
109 Difco Laboratories). Media were supplemented with 40 µg/ml kanamycin and 25 µg/ml
110 chloramphenicol (Eurobio), when required. *C. difficile* strains (Table 1) were grown in an anaerobic
111 chamber at 37°C in Brain Heart Infusion (BHI, Difco Laboratories) for the MIC experiments and in
112 Tryptone Yeast extract (TY) medium (Difco Laboratories) for the other experiments. The following
113 antibiotics: thiamphenicol (15 µg/ml, MP Biomedical), supplement (composed of 250 µg/ml of D-
114 cycloserine and 8 µg/ml of cefoxitin, Oxoid), erythromycin (10 µg/ml), vancomycin (0.5, 1 or 1.5
115 µg/ml) and tetracycline (5 µg/ml) were added when required. All antibiotics were added at the start
116 of the experiments, except in the experiment using *gusA* as reporter (see paragraph β-glucuronidase
117 activity and induction with different substances). The MICs of vancomycin were determined by E-
118 test (bioMérieux) on BHI agar at least in four independent experiments similarly to Ammam *et al.*
119 [13]. To determine the other MICs, strains were grown overnight in TY liquid medium. Cultures were
120 then diluted in medium to obtain OD_{600nm} = 0.05, and different concentrations of lysozyme or
121 antibiotics were added in 96-well plates. Positive wells were considered as a visual growth after 20
122 hours of incubation. MIC of a given condition was determined as the last well for which no growth
123 was observed.

124

125

126 **DNA manipulations**

127 Plasmid extraction, endonuclease digestion, ligation and agarose gel electrophoresis were carried out
128 as described by Maniatis *et al.* [14]. Polymerase chain reaction (PCR) for cloning DNA fragments
129 was carried out with high-fidelity *Phusion* DNA polymerase (Finnzymes) and for screening with
130 Red'y'Gold Mix (Eurogentec). Restriction enzymes were used according to the manufacturer's
131 instructions (Neb Biolabs). All cloning steps were verified using restriction analysis and sequencing.
132 The *asnB* clostron mutant was constructed as described by Heap *et al.* using primers presented in
133 Table 2 [15]. Clostron was amplified using primers TC107, TC108, TC109 and EBS universal and
134 cloned in the pCR-Blunt vector (Invitrogen), giving rise to pTC86. Clostron was then sub-cloned into
135 pMTL007C-E5 and sequenced, giving rise to pFA48. After conjugation in 630Δ*erm*, mutants were
136 screened using PCR. Intron was introduced in base pair position 790 of *asnB*.

137 *asnB* and *asnB2* were cloned in the pCR-Blunt vector (Invitrogen) using TC117/TC114 and
138 TC116/TC118 primers, respectively. Each gene was subcloned into pRPF185 using BamHI and SacI
139 giving rise to pTC88 and pTC87, respectively. pTC116 was constructed using the Gibson assembly
140 protocol (New England Biolabs) and the primers TC212 to TC217 where the *asnB* promoter and *gusA*
141 reporter gene [16] were cloned in the pMTL84121 plasmid [17].

142 pET plasmids allowing expression of *asnB* and *asnB2* genes in *E. coli* were constructed as follows.
143 PCR primers pETasnB-F and pETasnB-R were used to amplify the 1,584-bp *asnB* gene from the
144 *C. difficile* strain 630 chromosome and the resulting material was treated with BspHI and BamHI
145 and ligated between the compatible sites NcoI and BglIII of pET2160 vector (a pET21d derivative)
146 [18], generating the pMLD405 plasmid. Similarly, the 1,875-bp *asnB2* gene was amplified using
147 primers pETasnB2-F and pETasnB2-R and the PCR product was cleaved by NcoI and BglIII and
148 cloned between the corresponding sites of pET2160 vector, yielding the pMLD389 plasmid.
149 Sequences of cloned inserts were controlled by DNA sequencing.

150

151 **Transcription analysis**

152 Real-time quantitative RT-PCR (qRT-PCR) was performed as described in [13] using primers
153 described in Table 2. Relative expression levels of *asnB* and *asnB2* genes were measured using Pfaffl
154 mathematical model for relative quantification [19]. Comparisons between conditions were
155 performed using the Student's paired *t*-test. Differences were considered significant at $P \leq 0.05$.

156

157 **Peptidoglycan structure analysis**

158 *E. coli* BL21(DE3)(pLysS) cells (0.8-liter cultures) carrying the pMLD405 (*asnB*) plasmid,
159 or the empty vector pET2160, were grown exponentially at 37°C in 2YT-ampicillin medium. At an
160 OD_{600nm} of 0.2, 1 mM IPTG was added and incubation was continued for 2 h (final OD of 0.9-1.0).
161 Bacteria were harvested in the cold, washed with a cold 0.85% NaCl solution and then rapidly
162 suspended under vigorous stirring in 40 ml of a hot (95 to 100°C) aqueous 4% SDS solution for 1 h.
163 After standing overnight at room temperature, suspensions were centrifuged for 30 min at 200,000 ×
164 *g* and the pellets were washed several times with water. After final resuspension in 3 ml of water,
165 aliquots were hydrolyzed (16 h at 95°C in 6 M HCl) and analyzed with a Hitachi model 8800 amino
166 acid analyzer (ScienceTec). The peptidoglycan (PG) content was expressed in terms of its
167 characteristic constituents, muramic acid and diaminopimelic acid [20, 21]. The PG structure was

168 then determined by using the procedure of Glauner consisting in the analysis of the muuropeptides that
169 are generated following digestion of the polymer by a muramidase activity [22].

170 The purified PG preparations were treated with mutanolysin (Sigma) and the released fragments
171 (muuropeptides) were reduced with sodium borohydride and separated by HPLC on a 3- μ m ODS-
172 Hypersil column (4.6 \times 250 mm, Thermo Scientific), using a gradient of methanol (from 0 to 25% in
173 90 min) in 50 mM sodium phosphate, pH 4.5, at a flow rate of 0.5 ml/min. Peaks of muuropeptides
174 were detected at 207 nm and the main ones were collected, lyophilized and then desalted on the same
175 HPLC column using this time 0.05% trifluoroacetic acid and a gradient of methanol (0 to 25%) for
176 elution. They were identified by amino acid and amino sugar analysis and by MALDI-TOF mass
177 spectrometry.

178 **Pools of peptidoglycan nucleotide precursors.**

179 Cultures of the different *C. difficile* strains (0.5 liter) were made at 37°C in TY medium and were
180 stopped in exponential phase at an OD_{600nm} of 0.9. Cultures of the 630 Δ *erm* strain, the *asnB* mutant,
181 the complemented strain (630 Δ *erm* *asnB*::*erm* + pTC88 or pTC87 plasmids carrying *asnB* and *asnB2*,
182 respectively), and the 630 Δ *erm* *vanR*::*erm* and 630 Δ *erm* *vanG*::*erm* strains were grown in the
183 absence or presence of vancomycin to induce peptidoglycan precursor amidation. Tetracycline (5
184 μ g/ml) was added to the culture to induce *asnB* or *asnB2* expression when necessary. Thiamphenicol
185 (15 μ g/ml) was added when pTC88 or pTC87 were in the 630 Δ *erm* or in the *asnB* mutant. *E. coli*
186 BL21(DE3)(pLysS) cells (0.8-liter cultures) carrying either the pMLD405 (*asnB*) or pMLD389
187 (*asnB2*) plasmid, or the pET2160 vector, were grown at 37°C in 2YT medium and expression of these
188 genes was induced for 2 h with 1 mM IPTG, as described above. Then, cultures were rapidly chilled
189 to 0°C and cells were harvested in the cold. The extraction, separation and quantification of PG
190 nucleotide precursors was performed as described previously [20, 23]. The final separation step of
191 the different UDP-MurNAc-peptides was performed as follows: aliquots were applied on a
192 μ Bondapak C18 HPLC column (7.8 \times 300 mm) and elution was done at a flow rate of 3 ml/min with
193 50 mM ammonium formate for 15 min at pH 3.35, followed by a gradient of pH, from 3.35 to 4.75,
194 applied between 15 and 50 min. Nucleotide precursors were identified on the basis of their retention
195 time, as compared to standards. Their identity was confirmed by analysis of their amino acid and
196 amino sugar composition, as well as by MALDI-TOF mass spectrometry.

197

198 **MALDI-TOF mass spectrometry analyses**

199 Positive and negative spectra were recorded in the reflectron mode with delayed extraction on a
200 Perseptive Voyager-DE STR instrument (Applied Biosystems) equipped with a 337-nm laser. PG
201 precursors and mucopeptides (1 μ l of 50-100 pmol/ μ l solution in water) were deposited on the plate,
202 followed by 2,5-dihydroxybenzoic acid (1 μ l at 10 mg/ml in 0.1 M citric acid). After evaporation of
203 water, spectra were recorded at an acceleration voltage of \pm 20 kV and an extraction delay time of
204 200 ns. External calibration was performed using the calibration mixture 1 of the SequazimeTM
205 peptide mass standards kit (Applied Biosystem) in the positive mode, or a mixture of UDP-MurNAc,
206 UDP-MurNAc-dipeptide and UDP-MurNAc-pentapeptide in the negative mode.

207

208 **β -glucuronidase activity and induction with different substances**

209 *C. difficile* 630 strains carrying pRPF185 or pTC116 were grown until $0.5 < OD_{600nm} < 0.6$ which
210 corresponds to the middle of the exponential phase and then incubated for 2 h with lysozyme
211 (10 mg/ml), NaCl (0.15 M), vancomycin (1 or 1.5 μ g/ml), taurocholate (0.1 %), polymyxin B (125
212 μ g/ml), ramoplanin (20 μ g/ml) or ampicillin (100 μ g/ml). β -glucuronidase activity assay was
213 performed according to the protocol described by Dupuy and Sonenshein [24], except for the lysis
214 which was performed in Z buffer (60 mM Na₂HPO₄, pH 7, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM
215 MgSO₄ and 50 mM 2-mercaptoethanol) for 40 min at 37°C.

216

217 **Statistics used in the study**

218 Comparisons between conditions were performed using the Student's paired *t*-test. Differences were
219 considered significant at $P \leq 0.05$.

220

221 RESULTS AND DISCUSSION

222 *asnB* expression is induced in the presence of vancomycin

223 Two genes in the *C. difficile* genome [25] were annotated as two putative asparagine synthetase genes
224 *asnB* (CD630_03910) and *asnB2* (CD630_16320). *In silico* analysis, using pfam database
225 (<https://pfam.xfam.org>), showed that both AsnB and AsnB2 proteins harboured a putative glutamine
226 amidotransferase domain (GATase 7) at the N-terminal position and an asparagine synthetase domain
227 at the C-terminal part. Both proteins showed 25.5% identity and 73.5% of similarity. No predicted
228 active sites were found using the pfam database. Interestingly, *asnB2* was separated by three genes
229 from the *vanG_{Cd}* locus (Fig. 1) while *asnB* gene was far away, from approximately 1421 kb (Fig. 1).
230 Both genes appeared well conserved among the *C. difficile* genomes described by Kurka *et al.* [26],
231 even in the strains that did not harbour the *vanG_{Cd}* cluster. To determine whether *asnB* and/or *asnB2*
232 were expressed in the presence of vancomycin [13], we analyzed and compared their expression by
233 qRT-PCR following growth of *C. difficile* in the presence or absence of this antibiotic (Fig. 2). Only
234 *asnB* expression was increased (≈ 70 fold, $p \leq 0.0005$) in the presence of vancomycin (1.5 $\mu\text{g/ml}$).
235 However, it did not change in the presence of other antibiotics blocking the transglycosylation step
236 of PG synthesis, such as teicoplanin (0.025 $\mu\text{g/ml}$) and moenomycin (0.1 $\mu\text{g/ml}$), suggesting a
237 specific vancomycin-dependent induction mechanism of *asnB* gene expression. In contrast, no
238 statistical significant difference was observed in the expression level of *asnB2* when *C. difficile* was
239 grown in the presence of either of these antibiotics (Fig. 2). The observation that *asnB2* expression
240 was not induced by vancomycin and that no amidation of PG precursors could be detected in the
241 absence of this antibiotic (see below and [13]) clearly suggested that AsnB, but not AsnB2, may be
242 involved in PG precursor amidation in *C. difficile* in these conditions.

243

244 AsnB mediates PG amidation in *E. coli*

245 The potential role of *C. difficile* AsnB and/or AsnB2 asparagine synthetases in PG amidation was
246 first questioned by analyzing the effects of the heterologous expression of these two genes in a *E. coli*
247 cell background. The induction of AsnB overexpression using the strain BL21(DE3)(pLysS)
248 (pMLD405) in exponentially-growing cells was followed by an arrest of growth about 2 h later and
249 the OD_{600nm} then slowly decreased, suggesting a loss of cell integrity and potential block in the PG
250 biosynthesis pathway. When the AsnB2 variant was similarly overexpressed (in BL21(DE3)(pLysS)
251 (pMLD389), no such effects on the bacterial growth were observed (data not shown). The pools of
252 the soluble UDP-MurNAc-peptide precursors were extracted from these cells and analyzed by HPLC,

253 as depicted in Fig. 3. As usually observed, the main precursor detected in control wild-type *E. coli*
254 BL21 cells was UDP-MurNAc-pentapeptide (UDP-MurNAc-L-Ala- γ -D-Glu-*meso*-DAP-D-Ala-D-
255 Ala), i.e. the last cytoplasmic precursor of the PG pathway, that does not contain any amidated residue
256 (Fig.3A). Overexpression of *asnB* in the host strain, using plasmid pMLD405, resulted in a dramatic
257 decrease of the pool of this precursor and in the appearance of a new peak on the HPLC profile, eluted
258 at a higher retention time and corresponding to a *meso*-DAP_{NH₂}-containing, amidated form of UDP-
259 MurNAc-pentapeptide (Fig. 3B), a compound that normally does not exist in *E. coli*. Interestingly,
260 when expressing *ltsA* from *C. glutamicum* in *E. coli*, the growth was stopped and this phenotype was
261 attributed to an interference with peptidoglycan biosynthesis [3], suggesting that the expression of
262 *asnB* in *E. coli* acts in similar manner. This modified precursor remained nearly undetectable in
263 *asnB2*-overexpressing BL21(DE3)(pLysS)(pMLD389) cells (Fig. 3C), suggesting that, in *E. coli*,
264 only AsnB was responsible for this PG precursor modification. To confirm that the amidation found
265 on the soluble precursors was also incorporated in the mature PG polymer, an analysis of the PG
266 structure was then performed comparing *E. coli* cells expressing the AsnB protein (pMLD405
267 plasmid) and the strain carrying the empty control (pET2160). As shown in Fig. 4, the analysis of the
268 PG from AsnB-expressing cells showed additional peaks of monomers and dimers with increased
269 retention times and hydrophobicity as compared to the control muropeptides, consistent with a
270 modification by amidation of carboxyl groups present in their peptide chains. The latter muropeptides
271 were purified, characterized (composition and mass spectrometry analysis) and shown to have a
272 molecular mass by 1 dalton (for monomers) or either 1 or 2 daltons (for dimers) lower as compared
273 to the classical muropeptides (Table 3). We previously analysed this modification [13] and were able
274 to identify it as a modification of *meso*-DAP into *meso*-DAP_{NH₂}. It confirmed that the new peaks 3,
275 5 and 6 detected in Fig. 4 consisted in an amidated form of the main monomer (GlcNAc-MurNAc-L-
276 Ala- γ -D-Glu-*meso*-DAP_{NH₂}-D-Ala-D-Ala, designated as “Tetra_{NH₂}”) and of mono- and di-amidated
277 forms of the corresponding dimer (“Tetra_{NH₂}-Tetra” and “Tetra_{NH₂}-Tetra_{NH₂}”, Tetra being GlcNAc-
278 MurNAc-L-Ala- γ -D-Glu-*meso*-DAP-D-Ala), respectively. It is to note that the pool level of UDP-
279 MurNAc-tripeptide was comparatively very low (< 5 nmoles /-liter culture extract, not shown) and
280 no significant peak corresponding to amidated UDP-MurNAc-tripeptide could be observed in HPLC
281 analyses of AsnB-overexpressing *E. coli* cell extracts. These results thus highlighted the
282 amidotransferase activity of the *C. difficile* AsnB protein and its essential role in this PG structure
283 modification.
284

285 **AsnB is responsible for *C. difficile* PG precursor amidation**

286 To confirm that *asnB* was responsible for PG precursor amidation in *C. difficile*, an *asnB* mutant of
287 *C. difficile* 630 Δ *erm* was constructed. In contrast to *L. plantarum* and *C. glutamicum* species where
288 the deletion of *asnB2* and *ltsA* genes resulted in defective mutants with altered growth and cell
289 morphology [2, 3], the *C. difficile* *asnB* mutant did not exhibit any particular defect when grown in
290 BHI (data not shown). Similarly, no difference in growth kinetics was observed in comparison to the
291 parental strain. The pools of PG precursors were extracted and analyzed from the wild-type *C. difficile*
292 630 Δ *erm* strain, the *asnB* mutant and the complemented mutant following growth in the presence or
293 not of a sub-inhibitory concentration of vancomycin (1.5 μ g/ml) (Fig. 5). We earlier reported that the
294 induction of the *C. difficile* *vanG*_{Ca} cluster by vancomycin resulted both in a partial replacement of
295 D-Ala by D-Ser at the 5th position of the UDP-MurNAc-pentapeptide peptide chain and in a partial
296 amidation of *meso*-DAP residues in the PG precursors [13]. As shown here, UDP-MurNAc-
297 pentapeptide(Ser), UDP-MurNAc-pentapeptide(Ala) and UDP-MurNAc-tetrapeptide (resulting from
298 the degradation of pentapeptide by D,D-carboxypeptidase activities) coexisted and were detected in
299 both amidated and non-amidated forms in the wild-type 630 Δ *erm* strain content. In the presence of
300 vancomycin, the inactivation of *asnB* led to a total loss of amidation of all these precursors but did
301 not abolish the replacement of D-Ala by D-Ser (Table 4). Interestingly, complementation of the *asnB*
302 mutant by the pTC88 plasmid, in the presence of 5 μ g/ml of tetracycline as inducer, not only restored
303 the PG precursor amidation ability but also greatly increased its extent, > 90% of these precursors
304 being amidated in these conditions versus *ca.* 30% in the parental strains (Table 4). These data thus
305 confirmed the role of AsnB in the amidation of PG precursors in *C. difficile*. They also showed that
306 the vancomycin-induced replacement of D-Ala by D-Ser residues in UDP-MurNAc-peptide
307 precursors by *vanG* cluster-encoded enzyme activities was not strictly dependent on the amidation
308 status of these PG precursors. Interestingly, when the *asnB2* gene was similarly overexpressed in the
309 *asnB* mutant using the pTC87 plasmid, a low pool of amidated UDP-MurNAc-pentapeptide(Ala)
310 could be detected, suggesting that AsnB2 could catalyze the amidation of PG precursors to some
311 extent, although with a much lesser efficiency and a different substrate specificity, as no amidated
312 UDP-MurNAc-tetrapeptide was detected in these conditions. Altogether, these results suggested that,
313 in physiological conditions, AsnB was responsible for the PG precursor amidation observed in *C.*
314 *difficile* in the presence of vancomycin.

315

316 **Impact of PG precursor amidation on the resistance to lysozyme and antibiotics**

317 Vancomycin was tested against the 630 Δ *erm*(pMTL84121), 630 Δ *erm asnB::erm*(pMTL84121) and
318 *asnB*-overexpressing 630 Δ *erm*(pTC88) strains. No change in MIC was observed in the 630 Δ *erm*
319 *asnB::erm*(pMTL84121) strain in comparison with the 630 Δ *erm*(pMTL84121) parental strain. In
320 contrast, the overexpression of *asnB* increased the cell susceptibility to this antibiotic, reducing its
321 MIC from 2-3 to 1-1.5 μ g/ml (Table 5). This result suggested that a high level of precursor amidation
322 interfered with vancomycin resistance and this amidation may therefore contribute, at least to some
323 extent, to the lack of resistance observed for strains carrying the *vanG_{Ca}* operon [13]. Recently, it has
324 been shown that the constitutive expression of the *vanG_{Ca}* resistance operon in ribotype 027 *C.*
325 *difficile* clinical strains promotes vancomycin resistance [27]. It would be interesting to know the
326 extent of PG *meso*-DAP amidation in the absence and in the presence of vancomycin to evaluate the
327 impact of *asnB* in these strains.

328 In *B. subtilis*, the Δ *asnB* mutant was more sensitive to lysozyme and to antibiotics targeting different
329 steps of cell wall synthesis [5]. We therefore tested whether the *C. difficile asnB* mutant was more
330 sensitive to these compounds. The minimal inhibitory concentration (MIC) of lysozyme was similar
331 for the parental and the *asnB* mutant strains (3.125 μ g/ml). Since the overexpression of *asnB* induced
332 a large increase of the extent of PG precursor amidation, the MIC for lysozyme was also tested in
333 these conditions and again no difference in MIC value was observed. Similarly, all the strains were
334 tested for their resistance to penicillin and cefuroxime targeting cell wall synthesis, as well as for their
335 susceptibility to ciprofloxacin, fusidic acid and rifampicin as controls, and no differences were noted
336 (data not shown). These results suggested that the level of PG precursor amidation did not affect the
337 resistance to lysozyme or to the tested antibiotics.

338

339 ***asnB* is specifically induced in the presence of vancomycin**

340 RT-PCR analysis showed that *asnB* was co-transcribed with a small open reading frame (ORF)
341 CD630_03910 located just upstream (Fig. S1), suggesting that both genes formed an operon.
342 CD630_03910 encoded a putative 40-amino acid protein with unknown predicted function. The
343 putative promoter placed upstream from ORF CD630_03910 (arrow, Fig. 1) was cloned to control
344 the *gusA* gene as a reporter. In the absence of vancomycin, assay of β -glucuronidase in *C. difficile*
345 630 Δ *erm*(pTC116) compared to the control 630 Δ *erm*(pRPF185) showed no significant difference,
346 suggesting that *asnB* was not expressed. This result was similar to a previous study that reported that
347 no RNA was detected for *asnB* or *asnB2* when cells were grown in TY medium [28], suggesting that
348 both genes were not expressed. In contrast, a significant increase of this activity was observed in the

349 presence of vancomycin (Fig. 6). This was in agreement with the observed induction of *asnB* gene
350 expression as judged by qRT-PCR (Fig. S1). Furthermore, no significant β -glucuronidase activity
351 was detected in the presence of lysozyme, NaCl, taurocholate, polymyxin B, ramoplanin or
352 ampicillin. In addition, the fact that the PG precursors were found amidated when *C. difficile* was
353 grown at 0.5 μ g/ml vancomycin without leading to any growth defect, together with our results,
354 suggested that *asnB* was not regulated by a global surface regulation mechanism but rather
355 specifically by vancomycin. In addition, neither *asnB* nor *asnB2* genes were shown to be expressed
356 in an animal model [29].

357

358 **Expression of *asnB* is independent from *vanG_{Cd}* expression**

359 Since vancomycin specifically induced the expression of the *vanG_{Cd}-vanXY_{Cd}-vanT_{Cd}* operon through
360 the *vanRS* regulation operon, PG precursor amidation was also analyzed in *vanR* and *vanG* mutants
361 of *C. difficile* 630 Δ *erm* grown in the presence of vancomycin (Table 6). No difference was observed
362 between the parental and mutant strains in this regard, the PG precursors being similarly amidated in
363 all cases (Table 6). The unchanged level of amidation in the *vanR* mutant indicated that the two-signal
364 transduction system VanR/VanS was not involved in the regulation of *asnB*, suggesting that
365 vancomycin induced *asnB* independently from the *vanG_{Cd}* genes. Even though it appears unlikely, we
366 cannot exclude a potential cross-talk of VanS with another regulator. This result suggested that *asnB*
367 expression did not require the presence of the *vanG_{Cd}* resistance cluster.

368

369 **Conclusion**

370 In this paper, we found that AsnB was responsible for the PG precursor amidation and the subsequent
371 PG amidation observed in the presence of vancomycin [13]. AsnB2 could also potentially participate
372 to the PG amidation to some extent since, when overexpressed, a low level of amidation of the UDP-
373 MurNAc-pentapeptide(Ala) precursor was detected. However, in the presence of vancomycin, only
374 the expression of *asnB* was specifically induced. The mechanism by which vancomycin induces *asnB*
375 expression independently from the *vanG_{Cd}* cluster remains to be investigated. Moreover, the benefit
376 conferred to *C. difficile* by PG amidation remains to be elucidated.

377

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380

381 **Conflicts of interest**

382 The author(s) declare that there are no conflicts of interest

383

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387

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461

462

| Strain or plasmid | Genotype and relevant characteristics | Source or reference |
|---|---|---------------------|
| Strains | | |
| TG1 | K-12 <i>glnV44 (supE) thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(r_K⁻ m_K⁻) F'[traD36 proAB⁺ lacI^q lacZΔM15]</i> | Invitrogen |
| HB101 pRK24 | F ⁻ <i>Δ(gpt-proA) 62 leuB6 glnV44 ara-14 gal-K2 lacY1 Δ(mcrC-mrr) rpsL20 (str) xyl-5 mtl-1 recA13</i> , pRK24 | Invitrogen |
| BL21(DE3)(pLysS) | F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁻) gal dcm</i> (DE3) pLysS (Cam ^R) | Novagen |
| <i>C. difficile</i> 630Δ <i>erm</i> | A+B+, serotype C, Em ^S , Tc ^r , Rif ^S | |
| <i>C. difficile</i> 630Δ <i>erm asnB::erm</i> | Clostron mutant in CD630_03910 targeting the 790s site | This work |
| <i>C. difficile</i> 630Δ <i>erm vanR::erm</i> | Clostron mutant in CD630_16240 targeting the 45s site | [13] |
| <i>C. difficile</i> 630Δ <i>erm vanG::erm</i> | Clostron mutant in CD630_16260 targeting the 490s site | [13] |
| Plasmids | | |
| pMTL84121 | Cloning vector used for Gibson assembly to construct pTC116 | [17] |
| pCR-Blunt | Opened cloning vector | Invitrogen |
| pRPF185 | Circular cloning vector, <i>catP</i> , <i>Ptet</i> , <i>gusA</i> | [16] |
| pTC81 | pCR-Blunt derived plasmid for <i>asnB</i> cloning | This work |
| pTC82 | pCR-Blunt derived plasmid for <i>asnB2</i> cloning | This work |
| pTC87 | pRPF185 derived plasmid for <i>asnB2</i> overexpression | This work |
| pTC88 | pRPF185 derived plasmid for <i>asnB</i> overexpression | This work |
| pTC116 | pMTL84121 derived plasmid for P _{<i>asnB</i>} β-glucuronidase assay | This work |
| pMTL007C-E5 | pCD6 FRT-flanked <i>ermB</i> RAM | [15] |
| pTC86 | pCR-Blunt containing the intron targeting <i>asnB</i> | This work |
| pFA48 | pMTL007C-E5 :: <i>asnB</i> -790s | This work |
| pET2160 | pET21d derivative, cloning vector, Ap ^R | [18] |
| pMLD405 | pET2160 derived plasmid for <i>asnB</i> overexpression | This work |
| pMLD389 | pET2160 derived plasmid for <i>asnB2</i> overexpression | This work |

Table 1 : Strains and plasmids used in this study

| Primer name | Sequence 5' - 3' | Description |
|-------------|---|-----------------------------------|
| FA59 | AGCTTCTCTATGGTTCATCTATTCGT | <i>asnB</i> -F qRTPCR |
| FA60 | TCTTCATTTAACTCTTTCACAAATCCT | <i>asnB</i> -R qRTPCR |
| FA63 | CAGCAGGTCACTATTTTAGGGTAGA | <i>asnB2</i> -F qRTPCR |
| FA64 | ATTTCTTATATCATCCTCGTTCACA | <i>asnB2</i> -R qRTPCR |
| FA15 | TCCATCTATTGCAGGGTGGT | <i>polIII</i> qRTPCR |
| FA16 | CCCAACTCTTCGCTAAGCAC | <i>polIII</i> qRTPCR |
| TC107 | AAAAAAGCTTATAATTATCCTTAAAAGACATTGGAGTGCGCCAGATAGGGTG | <i>asnB</i> -790s-IBS primer |
| TC108 | CAGATTGTACAAATGTGGTGATAACAGATAAGTCATTGGAACAACTTACCTTTCTTT GT | <i>asnB</i> -790s-EBS1d primer |
| TC109 | TGAACGCAAGTTTCTAATTTTCGGTTTCTTTCCGATAGAGGAAAGTGTCT | <i>asnB</i> -790s-EBS2 primer |
| EBS | CGAAATTAGAACTTTCGTTTCAGTAAAC | EBS Universal primer |
| TC114 | GGATCCTTAACATTTTACAAAATAGATTTTCATACCAC | pTC81 construction |
| TC116 | GGATCCTTAATACTCGACATTTTTATGCTCAATAAATAATTG | pTC82 construction |
| TC117 | GAGCTCGAAAGGAGGTTTATTTAAAATGATAAATTTAGAAGAAAAGTATTTAACACG | pTC81 construction |
| TC118 | GAGCTCGAAAGGAGGTTTATTTAAAATGGACGGCAATATTTCAATTTATTATAAAGG | pTC82 construction |
| TC212 | CCATATGACCATGATTACGAATTCGAGCTCGAGTAAATTACAGCAATAGAGTGAATTA C | pTC116 construction |
| TC213 | GTTGGTAATATAAAGGGGGCAATATTTATGTTACGTCCTGTAGAAACCCCAACCCG | pTC116 construction |
| TC214 | GAAAAACCGCAGCAGGGAGGCAAACAATGAGGATCCTCTAGAGTCGACGTCACGCGT CC | pTC116 construction |
| TC215 | GTAATTCACTCTATTGCTGTAATTTACTCGAGCTCGAATTCGTAATCATGGTCATATGG | pTC116 construction |
| TC216 | CGGGTTGGGGTTTCTACAGGACGTAACATAAATATTGCCCCCTTTATATTACCAAC | pTC116 construction |
| TC217 | GGACGCGTGACGTCGACTCTAGAGGATCCTCATTGTTTGCCCTCCCTGCTGCGGTTTTTC | pTC116 construction |
| pETasnB-F | CGCGTCATGATAAATTTAGAAGAAAAGTATTTAACACG | pMLD405 construction |
| pETasnB-R | GCGCGGATCCTTAACATTTTACAAAATAGATTTTCATACC | pMLD405 construction |
| pETasnB2-F | CGCGCCATGGACGGCAATATTTCAATTTATTATAAAGGTG | pMLD389 construction |
| pETasnB2-R | CGCGAGATCTTAATACTCGACATTTTTATGCTCAATAAATAATTG | pMLD389 construction |

Table 2: Primers used in the study

| Peak no. (see Fig. 4) | Muropeptides | MALDI-TOF m/z $[M + H]^+$ | Calculated monoisotopic molecular mass |
|--|--|--------------------------------|---|
| Wild-type <i>E. coli</i> strain | | | |
| Monomers | | | |
| A | Tri | 871.4 | 870.4 |
| B | Tetra(Gly) | 928.5 | 927.4 |
| C | Tetra | 942.7 | 941.4 |
| Dimers | | | |
| D | Tetra-Tri | 1795.4 | 1793.8 |
| E | Tetra-Tetra | 1866.1 | 1864.9 |
| AsnB-expressing <i>E. coli</i> strain | | | |
| Monomers | | | |
| 1 | Tri | 871.6 | 870.4 |
| 2 | Tetra | 942.6 | 941.4 |
| 3 | Tetra _(NH₂) | 941.6 | 940.4 |
| Dimers | | | |
| 4 | Tetra-Tetra | 1866.2 | 1864.9 |
| 5 | Tetra _(NH₂) -Tetra | 1865.4 | 1863.9 |
| 6 | Tetra _(NH₂) -Tetra _(NH₂) | 1864.4 | 1862.9 |

Table 3: PG muropeptides in *E. coli*

| | Nanomoles/total extract (percentage) | | | | | |
|--|--|--|---------------------------------|---|--|--|
| | UDP- MurNAc- pentapeptide (Ser) | Amidated UDP- MurNAc- pentapeptide (Ser) | UDP- MurNAc- tetrapeptide | Amidated UDP- MurNAc- tetrapeptide | UDP- MurNAc- pentapeptide (Ala) | Amidated UDP- MurNAc- pentapeptide (Ala) |
| 630 Δ <i>erm</i> + 1.5 μ g/ml vancomycin | 31 (17.7) | 11 (6.3) | 24 (13.7) | 7 (4) | 65 (37.1) | 37 (21.1) |
| 630 Δ <i>erm asnB::erm</i> + 1.5 μ g/ml vancomycin | 76 (40.9) | 0 (0) | 34 (18.3) | 0 (0) | 76 (40.9) | 0 (0) |
| 630 Δ <i>erm asnB::erm</i> + pTC88 + 1.5 μ g/ml vancomycin + 5 μ g/ml tetracycline | 8 (3.8) | 86 (41.1) | 0 (0) | 23 (11) | 6 (2.9) | 86 (41.1) |
| 630E:: <i>asnB</i> + pTC87 (<i>asnB2</i>) + 5 μ g/ml tetracycline | 0 (0) | 0 (0) | 25 (18.8) | 0 (0) | 85 (63.9) | 23 (17.3) |
| 630 Δ <i>erm</i> + pTC88 (<i>asnB</i>) + 5 μ g/ml tetracycline | 0 (0) | 0 (0) | 6 (5.1) | 17 (14.5) | 4 (3.4) | 90 (76.9) |

Table 4: Amounts (in nmol) of purified *C. difficile* PG precursors
Percentage of each peptidoglycan precursor is presented between parentheses

| <i>C. difficile</i> | vancomycin MIC ($\mu\text{g/ml}$) |
|---|-------------------------------------|
| 630 Δ <i>erm</i> + pMTL84121 (empty) | 2-3 |
| 630 Δ <i>erm asnB::erm</i> + pMTL84121 | 2-3 |
| 630 Δ <i>erm</i> + pTC88 (<i>asnB</i>) | 1-1.5 |

Table 5: Vancomycin MIC

MIC determination were performed at least 4 times. The range corresponds to the observed value in the different experiments.

| | UDP- MurNAc- pentapeptide (Ser) | Amidated UDP- MurNAc- pentapeptide (Ser) | UDP- MurNAc- pentapeptide (Ala) | Amidated UDP- MurNAc- pentapeptide (Ala) |
|--|--|--|--|--|
| 630 Δ <i>erm</i> + 1.5 $\mu\text{g/ml}$ vancomycin | 75 (52.8) | 19 (13.4) | 39 (27.5) | 9 (6.3) |
| 630 Δ <i>erm::vanR</i> + 1.5 $\mu\text{g/ml}$ vancomycin | 0 (0) | 0 (0) | 206 (94.1) | 13 (5.9) |
| 630 Δ <i>erm::vanG</i> + 1.5 $\mu\text{g/ml}$ vancomycin | 0 (0) | 0 (0) | 178 (94.2) | 11 (5.8) |

Table 6: Amounts (in nmol) of PG precursors purified from *C. difficile* 630 Δ *erm* and *C. difficile* 630 Δ *erm vanG_{cd}* mutants in the presence of 1.5 $\mu\text{g/ml}$ vancomycin. The percentage of each peptidoglycan precursor with respect to the total of the presented peptidoglycan precursors is shown between parentheses.

Figure legends

Figure 1. Schematic representation of *asnB* and *asnB2* loci in *C. difficile* 630 Δ *erm*

(A) The ORFs *bglA* (CD630_03890), *bglG* (CD630_03900), CD630_03901, *asnB* (CD630_03910), CD630_03920 and CD630_03930 are predicted to encode a 6-phospho- β -glucosidase, a transcription anti-terminator, a PTS operon, a conserved hypothetical protein, an asparagine synthetase (glutamine-hydrolyzing), a radical SAM-family protein and a putative membrane protein, respectively.

(B) The ORFs *vanR*_{Cd} (CD630_16240) and *vanS*_{Cd} (CD630_16250), *vanG*_{Cd} (CD630_16260), *vanY*_{Cd} (CD630_16270), *vanT*_{Cd} (CD630_16280), CD630_16290, CD630_16300, CD630_16310 and *asnB2* (CD630_16320) encode a regulator and a sensor of a two-component signal transduction system, a D-alanine:D-serine ligase, a D-alanyl-D-alanine carboxypeptidase, an alanine/serine racemase, a hypothetical protein, a membrane protein, a superoxide dismutase and a putative asparagine synthetase, respectively. The *asnB* and *asnB2* genes are highlighted with dots. The arrow represents the putative promoter of the CD3901-*asnB* operon.

Figure 2. *asnB* and *asnB2* expression in *C. difficile* 630 Δ *erm*

asnB (A, B and C) and *asnB2* (D, E and F) transcripts were quantified by qRT-PCR in the presence of vancomycin (Vanco, A and D), teicoplanin (Teico, B and E) and moenomycin (Moeno, C and F). *C. difficile* was grown in TY or TY containing vancomycin (1.5 μ g/ml), teicoplanin (0.025 μ g/ml) and moenomycin (0.1 μ g/ml) and the culture was harvested at OD_{600nm} = 0.8 which corresponds to the middle of exponential phase of growth. In these conditions, the antibiotic concentrations are sublethal for *C. difficile* [13]. Expression value of *asnB* and *asnB2* in the absence of antibiotics was arbitrarily fixed to 1. Normalized fold expression is defined as the ratio between the qRT-PCR expression values obtained with antibiotic divided by the qRT-PCR expression values obtained without antibiotic. The mean values from three independent experiments are shown. Error bars represent standard deviations. * represents a significant difference in expression ($P \leq 0.0005$).

Figure 3. Analysis of the pools of *E. coli* PG precursors

asnB and *asnB2* genes were overexpressed in the *E. coli* BL21 strain using the pMLD405 and pMLD389 plasmids, respectively. PG precursors in these strains (B: +AsnB and C: +AsnB2 respectively) and the *E. coli* BL21 carrying the pET2160 (A: WT) were extracted and purified, and aliquots were analyzed by HPLC on a column of μ Bondapak C18 as described in Materials and

Methods. Peaks corresponding to the nonamidated (UM-Penta) and amidated (UM-Penta_{NH2}) forms of the main soluble PG precursor are indicated by a black arrows. The empty arrows indicate the lack of detection of the UM-Penta_{NH2}.

Figure 4. Analysis of *E. coli* PG muuropeptides

The patterns of muuropeptides released following digestion (mutanolysin) of PG from the *E. coli* strain BL21(DE3)(pLysS) carrying the empty pET2160 vector (*E. coli* strain) or the pMLD405 plasmid (*asnB*-expressing *E. coli* strain) were analyzed by HPLC. The identity and molecular mass of the main muuropeptides present in peaks A to E and 1 to 6 are reported in Table 3.

Figure 5. Analysis of *C. difficile* PG precursor pools

C. difficile strains were grown exponentially and the pools of PG nucleotide precursors were extracted and analyzed by HPLC on a column of μ Bondapak C18 as described in Materials and Methods. The A to E HPLC profiles correspond to the following *C. difficile* strains: A, the wild-type 630 Δ *erm* strain grown in presence of vancomycin (1.5 μ g/ml); B, the *asnB* mutant (630 Δ *erm* *asnB*::*erm*) grown in presence of vancomycin (1 μ g/ml); C, the *asnB*-overexpressing *asnB* mutant (630 Δ *erm* *asnB*::*erm* + pTC88) grown in presence of vancomycin (1.5 μ g/ml) and tetracycline (5 μ g/ml); D, the *asnB2*-overexpressing wild-type strain (630 Δ *erm* + pTC87) grown in presence of tetracycline (5 μ g/ml); and E, the *asnB*-overexpressing wild-type strain (630 Δ *erm* + pTC88) grown in presence of tetracycline (5 μ g/ml). The position on the HPLC profiles of the main UDP-MurNAc-peptides, which were subsequently purified, quantified and characterized, are indicated by arrows: 1, UDP-MurNAc-pentapeptide(D-Ser); 2, UDP-MurNAc-tetrapeptide; 3, amidated UDP-MurNAc-pentapeptide(D-Ser); 4, amidated UDP-MurNAc-tetrapeptide; 5, UDP-MurNAc-pentapeptide(D-Ala); 6, amidated UDP-MurNAc-pentapeptide(D-Ala). The residue indicated in parentheses is that found at the 5th position of the peptide chain and when present, the amidation occurs on the free carboxyl group of the *meso*-DAP residue.

Figure 6. Drug effects on the *asnB* expression

The β -glucuronidase activity was measured in the 630 Δ *erm* carrying the pRPF185 plasmid (185, harbours *gusA* under the control of the tetracycline inducible promoter) or carrying pTC116 (116, harbours *gusA* under control of the *asnB* promoter). Each strain was grown until $0.5 < OD_{600nm} < 0.6$ (middle of exponential phase of growth) and then placed for 2 h in the presence of the medium only

(T-), 10 mg/ml lysozyme (L), 0.15 M NaCl (Na), 1 µg/ml vancomycin (V1) or 1.5 µg/ml (V1,5), 0.1% taurocholate (Tauro), 125 µg/ml polymyxin B (poly), 20 µg/ml ramoplanin (ramo) or 100 µg/ml ampicillin (amp). The β-glucuronidase activity was reported as Miller units. At this concentration, the use of the antimicrobials stopped the bacterial growth with no or a minimal lysis of *C. difficile*. * represents a difference of β-glucuronidase activity that is significantly different than the one of the 630Δerm pTC116 grown only in the medium ($P \leq 0.005$).

Figure S1. RT-PCRs from *C. difficile* 630Δerm grown in the absence or in the presence of vancomycin

Primers FA63 (CAGCAGGTCACTATTTTAGGGTAGA) and FA64 (ATTTCTTATATCATCCTCGTTCACA) were used to follow the expression of *asnB2* (lanes 1 to 4). RT-PCRs were performed using FA62 (GAGCAGATTAGTACTAATTCAAA) and FA60 (TCTTCATTTAACTCTTTCACAAATCCT) primers to follow expression of *asnB* and ORF CD630_03910 (lanes 4 to 8). RT-PCRs were performed with purified total RNA from *C. difficile* 630Δerm grown in the absence (lanes 3 and 5) or presence (lanes 4 and 6) of vancomycin (0.5 µg/ml). Negative controls were performed without the reverse transcription steps lanes 1 and 7 and positive controls were performed using genomic DNA lanes 2 and 8.