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Critical impact of peptidoglycan precursor amidation on the activity of L,D-transpeptidases from *Enterococcus faecium* and *Mycobacterium tuberculosis*

Flora Ngadjeua[#,[a]], Emmanuelle Braud[#,[b]], Saidbakhrom Saidjalolov[[b]], Laura Iannazzo[[b]], Dirk Schnappinger[[c]], Sabine Ehrt[[c]], Jean-Emmanuel Hugonnet[[a]], Dominique Mengin-Lecreulx[[d]], Delphine Patin[[d]], Mélanie Ethève-Quelquejeu[,[b]], Matthieu Fonvielle,*[,[a]] and Michel Arthur*[,[a]]*

Abstract: The bacterial cell wall peptidoglycan contains unusual L and D amino acids assembled in branched peptides. Insight into the biosynthesis of the polymer has been hampered by limited access to substrates and to suitable polymerization assays. Here we report the full synthesis of the peptide stem of peptidoglycan precursors from two pathogenic bacteria, *Enterococcus faecium* and *Mycobacterium tuberculosis*, and the development of a sensitive post-derivatization assay for their cross-linking by L,D-transpeptidases. Access to series of stem peptides showed that amidation of free carboxyl groups is essential for optimal enzyme activity, in particular the amidation of diaminopimelate (DAP) residues for the cross-linking activity of the L,D-transpeptidase Ldt_{Mt2} from *M. tuberculosis*. Accordingly, construction of a conditional mutant established the essentiality of AsnB indicating that this DAP amidotransferase is an attractive target for the development of anti-mycobacterial drugs.

Peptidoglycan is an essential and specific component of the bacterial cell wall.[1] The main role of this giant (cell-sized) macromolecule is to protect bacterial cells against the osmotic pressure of the cytoplasm. The peptidoglycan subunit consists of a disaccharide substituted by a pentapeptide stem (Figure 1), which is polymerized by glycosyltransferases for the elongation of the glycan chains (all glycosidic bonds are β-1,4) and by D,D-transpeptidases for cross-linking the glycan chains to each other.[2] The amide bond formed by the D,D-transpeptidases links the carbonyl of D-Ala at the 4th position of an acyl donor stem to the side-chain amino group at the 3rd position of an acyl acceptor stem (4→3 cross-link). These enzymes are the targets of β-lactam antibiotics such as penicillin.

The structure of peptidoglycan is generally conserved in bacteria belonging to the same species, but highly diverse between species, including members of the same genus.[3] The polymorphisms include the *N*-deacetylation, *O*-acetylation, and *N*-glycolylation of either or both GlcNAc and MurNAc. These modifications are mostly, if not exclusively, due to maturation of subunits containing the canonic GlcNAc-MurNAc motif. The most frequent variations in the sequence of the pentapeptide stem occur at the 3rd (*e.g.* L-Lys instead of diaminopimelic acid [DAP]) and at the 5th (*e.g.* D-Lac instead of D-Ala) positions (Figure 1b). Modifications of the pentapeptide stem involve the addition of a side-chain to the 3rd residue (*e.g.* D-*iso*Asn or Gly_5) and the amidation of the carboxyl groups (*e.g.* the α-carboxyl of D-Glu and D-*iso*Asp or the ε-carboxyl of DAP). A last source of polymorphism originates from the presence of 3→3 instead of 4→3 cross-links in mycobacteria (*e.g. Mycobacterium tuberculosis*) and in β-lactam-resistant mutants generated *in vitro* (*e.g. Enterococcus faecium*) (Figure 1a). The 3→3 cross-links are formed by transpeptidases of the L,D specificity, which cleave the L-Lys^3-D-Ala^4 or DAP^3-D-Ala^4 bond of an acyl donor containing a tetrapeptide stem and form L-Lys^3→L-Lys^3 or DAP^3→DAP^3 cross-links.

Variability in the peptidoglycan structure has been known for decades based on biochemical analyses of the cell wall.[3a] The corresponding enzymes have been described more recently, mostly because the complexity of their substrates has hampered their characterization.[4] Consequently, the biological significance of structural variability is poorly understood. It may involve various selective advantages.[3b] Resistance to vancomycin is mediated by replacement of D-Ala by D-Lac or D-Ser at the 5th position of peptide stems since this prevents binding of the drug to the precursors. Specific links in mature peptidoglycan are cleaved by hydrolytic enzymes produced by eukaryote hosts, such as lysozyme, which cleaves the MurNAc-GlcNAc β-1,4 bond, or by competing bacteria, such as lysostaphin, which cleaves glycyl-glycine bonds in the D-Ala^4→(Gly_5)-L-Lys^3 cross-bridges of *Staphylococcus aureus*. Variations in peptidoglycan structure are therefore potential defense mechanisms against hydrolytic enzymes.

Diversification of the structure of peptidoglycan precursors associated with speciation is thought to lead to a parallel evolution of the substrate specificity of the transpeptidases.[5] Genetic evidence in favor of this hypothesis is limited since impaired maturation of peptidoglycan precursors may have combined effects on numerous peptidoglycan biosynthetic steps in addition to transpeptidation. Scarce evidence has been provided by biochemical studies due to limited access to purified enzymes and substrates.[6] In this study, we have developed the chemical synthesis of peptidoglycan precursor analogues and a post-derivatization assay to directly assess the impact of amidation of peptidoglycan precursors on the formation of cross-links by purified L,D-transpeptidases from *E. faecium* and *M. tuberculosis*. We show that defects in amidation strongly impair the efficacy of these enzymes indicating that the amidotransférases[7] are attractive targets to develop alternatives to transpeptidase inhibition by β-lactam antibiotics in drug resistant bacteria.

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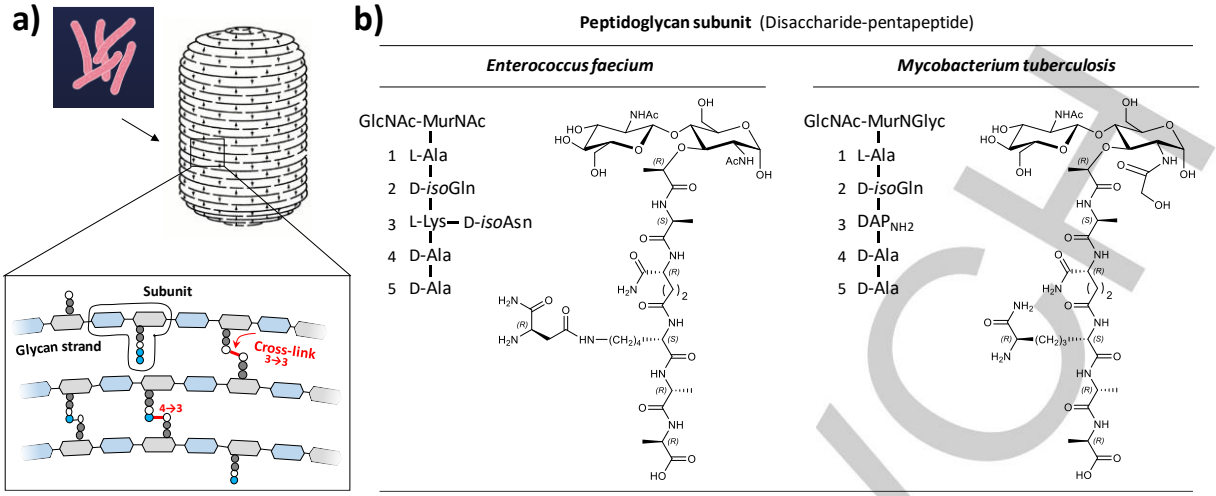
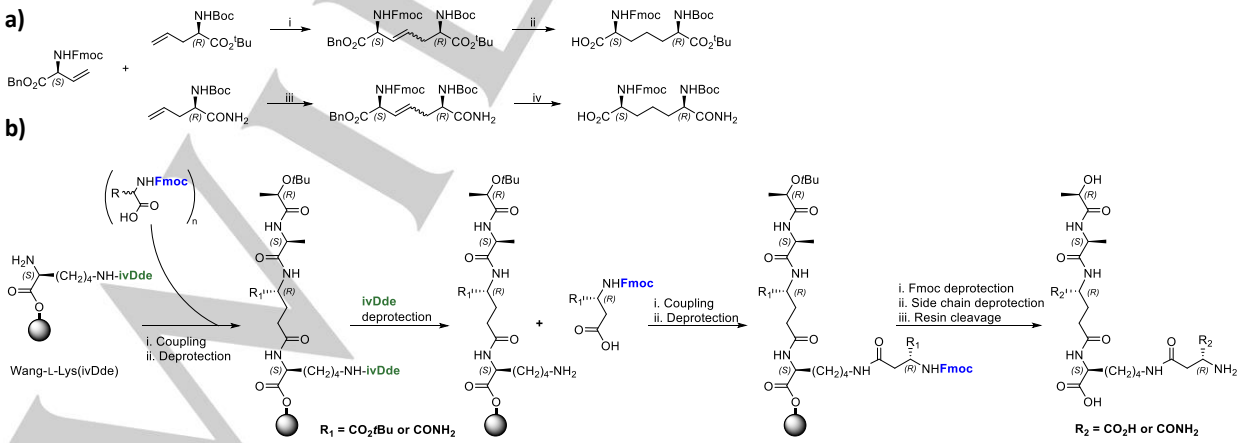


Figure 1. Peptidoglycan structure. a) Peptidoglycan is a giant mesh-like polymer that completely surrounds bacterial cells. b) Peptidoglycan is polymerized from disaccharide-pentapeptide subunits, which are assembled in the cytoplasm. MurNAc, *N*-acetyl muramic acid; MurNGlyc, *N*-glycolyl muramic acid; GlcNAc, *N*-acetyl glucosamine; D-*iso*Gln, D-*iso*-glutamine; D-*iso*Asn, D-*iso*-asparagine; DAP$_{NH2}$, diaminopimelic acid amidated at the ε position.

Peptidoglycan precursors contain unusual amino acids (D-*iso*Gln, D-Ala, D-*iso*Asn, amidated DAP), a non-peptide bond linking the γ-carboxyl of D-*iso*Gln to the α-amino group of L-Lys or DAP, and a side-chain linked to the ε-amino group of L-Lys (Figure 1b). Synthesis of peptides containing these amino acids have been previously reported using chemo-enzymatic methods,[8] chemical synthesis in solution,[9] or on solid phase.[10] For this study, we used the solid-support peptide synthesis strategy, which required access to non-commercial Fmoc-protected amino acids and the use of orthogonal protecting groups for the synthesis of branched peptides (Scheme 1). Synthesis of the Fmoc-protected DAP and amidated DAP has been performed using a cross-metathesis reaction as a key step (Scheme 1a).[11] Synthesis of the other protected amino acids is described in the Supplementary Material. Orthogonal Fmoc and 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl (ivDde) protecting groups were used for sequential assembly of the peptide stem and of the side-chain residue branched at the 3rd position, respectively (Scheme 1b). Using this approach 9 peptides mimicking peptidoglycan precursors have been synthesized and fully characterized.

A post-functionalization assay was developed to quantify the substrates and the product of the enzymatic cross-linking reaction catalyzed by Ldt$_{fm}$ from *E. faecium*, the prototypic enzyme of the L,D-transpeptidase family (Figure 2a). We took advantage of the presence of a single primary amine on these molecules to specifically introduce a 4-fluoro-7-nitrobenzofurazan group by nucleophilic aromatic substitution. This post-functionalization reaction applied to the crude enzymatic reaction converted the unreacted donor and acceptor substrates as well as the L,D-transpeptidation product (dimer) into fluorescent peptides. The post-functionalized peptides were separated by *rp*HPLC and detected by fluorescence (Figure 2b). Since the fluorescence yield may vary with the acetonitrile concentration required for elution and with the molecular environment of the fluorophore within the peptides, calibration curves were obtained for every substrate and every product (Figure 2c). For this purpose, each substrate and each product were individually post-functionalized with 4-fluoro-7-nitrobenzofurazan and known amounts of each fluorescent peptide were analyzed by *rp*HPLC. Access to sensitive determination of the peptides in the 10- to 200-pmol range provided a versatile method to determine the rate of the cross-linking reaction catalyzed by Ldt$_{fm}$.



Scheme 1. a) Synthetic scheme for the protected DAP and amidated DAP building blocks. i) 5% Grubb's II catalyst, CH_2Cl_2, RT, 12 h; ii) 3% PtO_2, H_2, $CH_2Cl_2/CH_3OH/H_2O$ (9/1/1), RT, 12 h; iii) 20% Grubb's II catalyst, 1,2-dichloroethane, 70°C, 48 h; iv) PtO_2, H_2, CH_3OH, 4 atm, RT, 18 h. b) Solid-phase-synthesis of the peptidoglycan precursor analogues using orthogonal Fmoc and ivDde protecting groups.

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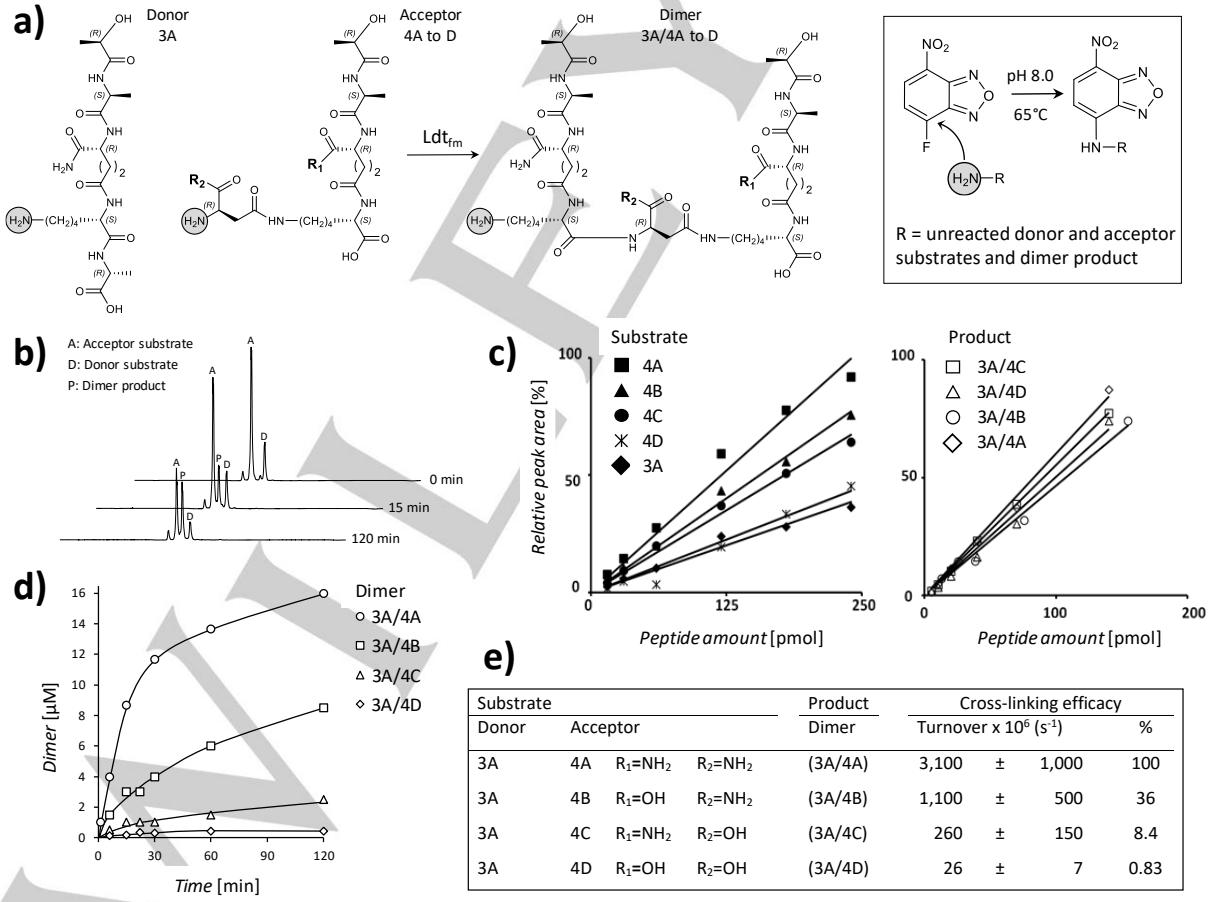
The reaction catalyzed by Ldt_{fm} involves two peptidoglycan precursors that act as an acyl donor and as an acyl acceptor (Figure 2a). For the donor, we used a linear tetrapeptide that cannot be used as an acyl acceptor since it does not harbor the D-*iso*Asn residue branched to L-Lys. Conversely, the peptides used as acyl acceptors cannot be used as acyl donors since they do not harbor the essential C-terminal D-Ala residue (D-Ala^4). To specifically assess the impact of amidation in the acceptor substrate, we tested a tetrapeptide donor containing a D-*iso*Gln residue (acyl donor 3A) and acceptors containing the four combinations of amidation of the D-*iso*Glu and D-*iso*Asp α-carboxyl groups (acyl acceptors 4A to 4D). The rate of formation of peptidoglycan dimers by Ldt_{fm} was the highest for the fully amidated acceptor, corresponding to the amidation status found in the peptidoglycan of the *E. faecium* host (Figures 2d and 2e). The lack of amidation of D-*iso*Gln had a moderate impact on the transpeptidase activity of Ldt_{fm} (36% residual activity). The impact of the lack of amidation was greater for the side-chain D-*iso*Asn residue (8.4% residual activity), whereas the combination of both modifications almost completely abolished the activity of Ldt_{fm} (0.83% residual activity). No transpeptidation product was observed for substrates fully lacking amidation both in the acyl donor and acceptor substrates (data not shown). In conclusion, amidation of the D-*iso*Glu and D-*iso*Asp α-carboxyl groups of the acyl acceptor substrate was essential for optimal formation of peptidoglycan cross-links by Ldt_{fm} *in vitro*. It has previously been shown that amidation of D-*iso*Glu is also required for effective formation cross-links by purified PBPs from *Streptococcus pneumoniae*.[12]

Our following objective was to evaluate the role of amidation of the α- and ε-carboxyl groups of D-*iso*Glu and DAP on the efficacy of formation of 3→3 cross-links by L,D-transpeptidases from *M. tuberculosis*. We based our analysis on Ldt_{Mt2} as a representative of the five L,D-transpeptidase paralogues produced by this species. Substrates containing the four combinations of amidation of the linear tetrapeptide stem of *M. tuberculosis* were synthesized and independently tested in the quantitative cross-linking assay (Figure 3). No cross-linked dimer was observed with substrates lacking amidation of DAP, D-*iso*Glu, or both. To assess the impact of the lack of amidation of peptidoglycan precursors on the growth of *M. tuberculosis*, we constructed mutants of strain H37Rv conditionally producing the DAP amidotransferase AsnB (Rv2201). This analysis showed that the amidation of the ε-carboxyl of DAP is required for growth of *M. tuberculosis* H37Rv.



e)

Substrate				Product	Cross-linking efficacy			
Donor	Acceptor			Dimer	Turnover x 10^6 (s^{-1})			%
3A	4A	R_1=NH_2	R_2=NH_2	(3A/4A)	3,100	±	1,000	100
3A	4B	R_1=OH	R_2=NH_2	(3A/4B)	1,100	±	500	36
3A	4C	R_1=NH_2	R_2=OH	(3A/4C)	260	±	150	8.4
3A	4D	R_1=OH	R_2=OH	(3A/4D)	26	±	7	0.83

Figure 2. Post-functionalization assay for the cross-linking activity of peptidoglycan transpeptidases. a) Reaction catalyzed by the L,D-transpeptidase Ldt_{fm}. Inset, post-functionalization reaction. b) Separation by *rp*HPLC of the post-functionalized substrates 3A and 4A and of the reaction product (dimer 3A/4A), which were detected by fluorescence (λ_{ex} = 470 nm; λ_{em} = 530 nm). c) Calibration curves for quantitative determination of the substrates (left panel) and reaction products (right panel). d) Kinetics of dimer synthesis by Ldt_{fm}. e) Impact of amidation of the acyl acceptor on the cross-linking efficacy of Ldt_{fm}.

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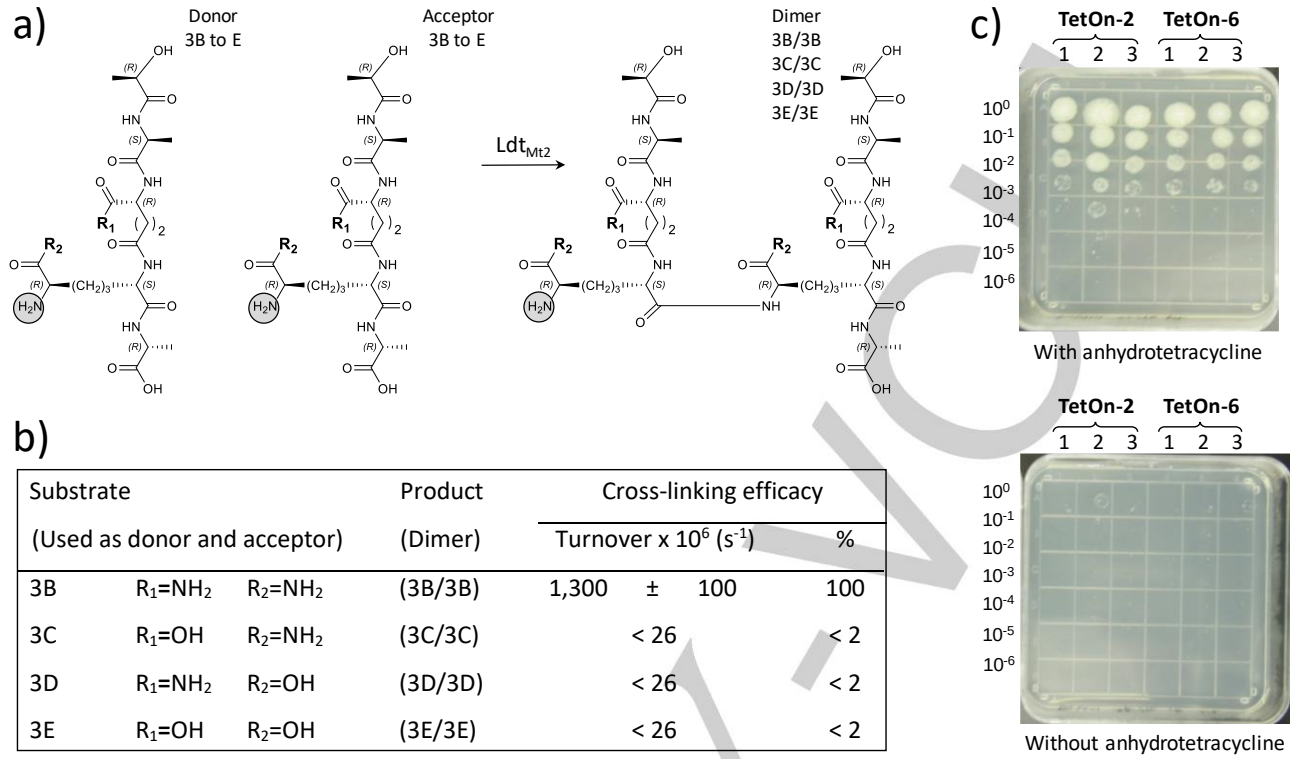

b)

Substrate (Used as donor and acceptor)			Product (Dimer)	Cross-linking efficacy Turnover x 10^6 (s^{-1})			%
3B	R_1=NH_2	R_2=NH_2	(3B/3B)	1,300	±	100	100
3C	R_1=OH	R_2=NH_2	(3C/3C)	< 26			< 2
3D	R_1=NH_2	R_2=OH	(3D/3D)	< 26			< 2
3E	R_1=OH	R_2=OH	(3E/3E)	< 26			< 2

Figure 3. Impact of impaired peptidoglycan precursor amidation on the activity of *M. tuberculosis* L,D-transpeptidase Ldt$_{Mt2}$. a) Transpeptidation reaction catalyzed by Ldt$_{Mt2}$. b) *In vitro* cross-linking activity of Ldt$_{Mt2}$. c) Impact of impaired amidation of DAP by AsnB (Rv2201) on the growth of *M. tuberculosis* H37Rv. Ten-fold dilutions of cultures of mutants Rv2201-TetON-2 and Rv2201-TetON-6 were spotted on the indicated media showing that depletion of AsnB in the absence of anhydrotetracycline prevents growth.

In conclusion, we have developed routes of synthesis of the peptide stems of peptidoglycan precursors that provide access to the full structural diversity found in bacteria, including the presence of DAP or L-Lys at the 3rd position, the presence or absence of a side-chain, and the amidation of carboxyl groups. We have also developed a sensitive assay for the detection of the products of the cross-linking reaction. Based on these new tools, functional analysis was focused on the impact of amidation of carboxyl groups in the D-*iso*Glu and DAP residues of peptidoglycan precursors on the activity of L,D-transpeptidases. For the first time, we directly establish that the diversification of the structure of peptidoglycan precursors is associated with a parallel evolution of the substrate specificity of cross-linking enzymes. Amidation of carboxyl groups was essential for the *in vitro* activity of L,D-transpeptidases from *E. faecium* and *M. tuberculosis*. Amidation of DAP was also essential for growth of *M. tuberculosis* indicating that the amidotransferase AsnB is a potential target for development of new drugs active on multi-drug resistant bacilli.

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Keywords: Amidation • Amidotransferase • *Mycobacterium tuberculosis* • Peptidoglycan • Transpeptidase

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Layout 1:

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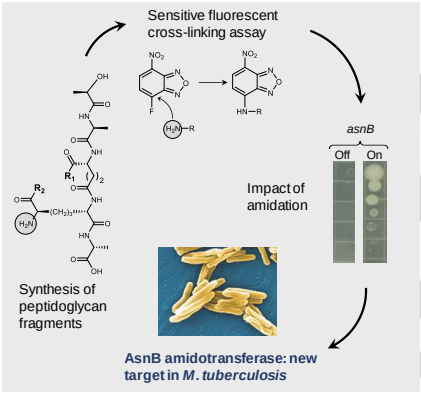
Synthetic routes to the peptide stem of peptidoglycan precursors and a sensitive fluorescent cross-linking assay were developed to assess the impact of structural variability on peptidoglycan polymerization. In the search for new targets for anti-mycobacterial drug development, this strategy was applied to the evaluation of diaminopimelate amidation in *Mycobacterium tuberculosis*, revealing the essential role of the AsnB amidotransferase for peptidoglycan transpeptidation both *in vitro* and *in vivo*.



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