Expanded View Figures

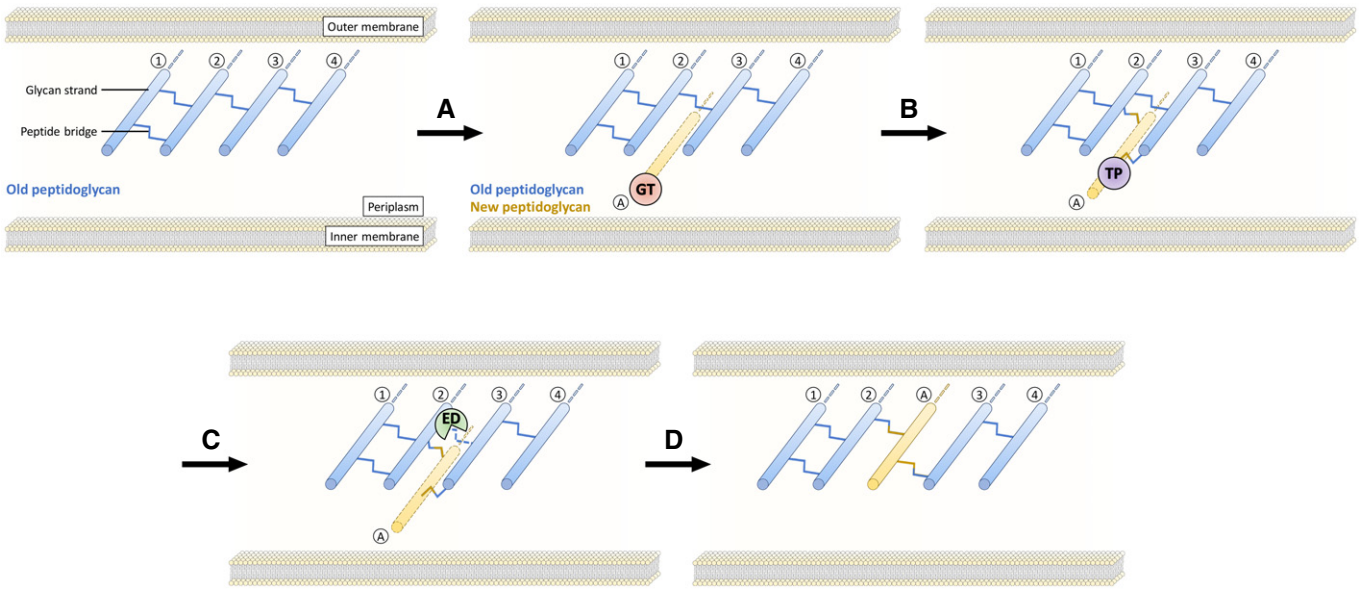

Figure EV1. Insertion of PG subunits into the growing PG network.

According to this model, one glycan strand (circled A) is polymerized by glycosyltransferases (GTs; step A) and attached to the pre-existing polymer (strands 2 and 3) by transpeptidases (TPs; step B). Hydrolysis of the cross-links connecting strands 2 and 3 by endopeptidases (EDs; step C) results in the expansion of the PG layer (step D). Of note, this model, which applies to the synthesis of the lateral wall, accounts for incorporation of new subunits sheltered from the cytoplasm osmotic pressure (De Jonge *et al*, 1989). There are other models in which whole glycan strands are removed (Höltje & Heidrich, 2001). All models predict that amide bonds should be cleaved for extension of the peptidoglycan network.

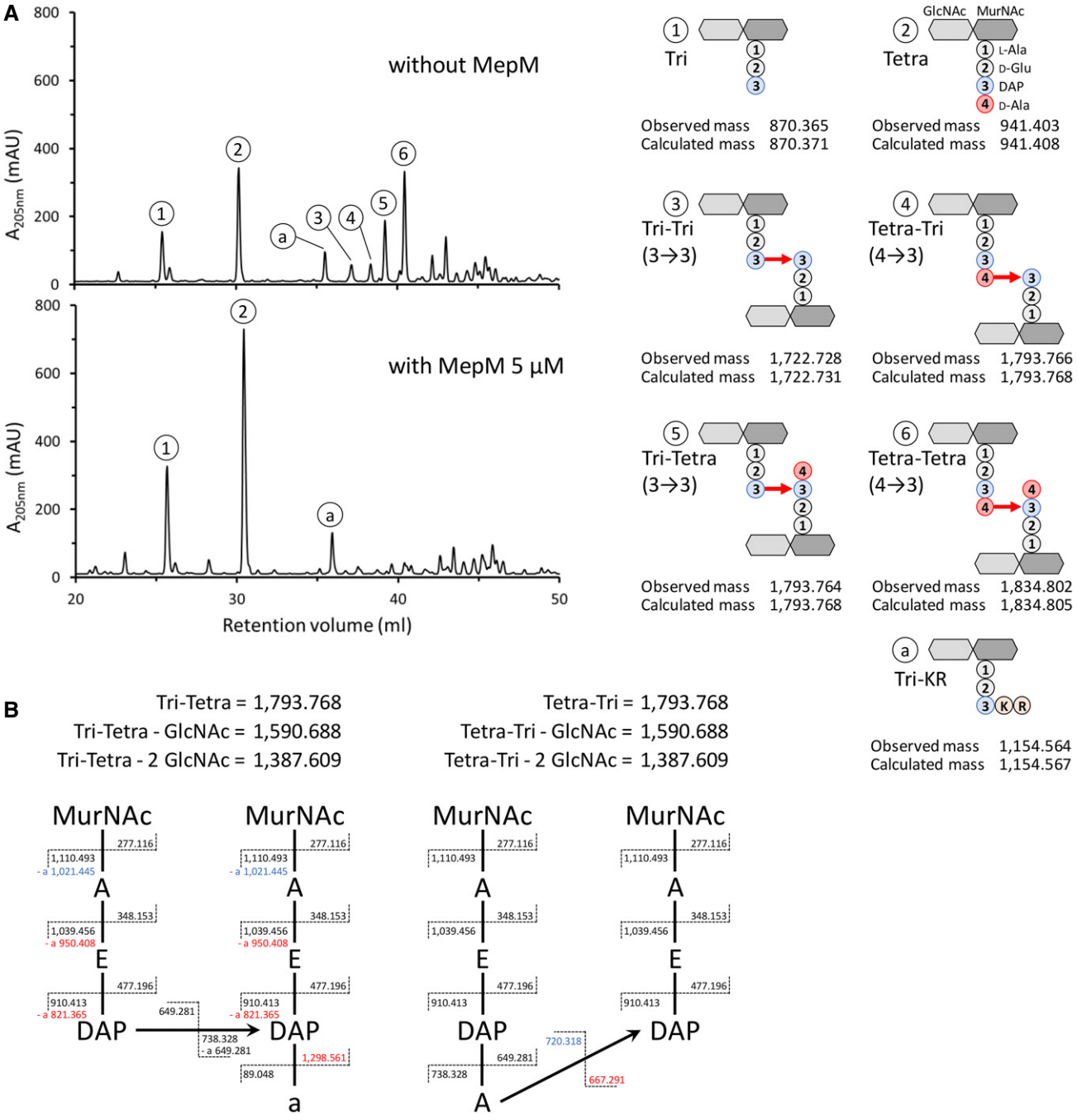

Figure EV2. Analysis of endopeptidases activity by mass spectrometry.

A Mass spectrometry analysis of muropeptides obtained by digestion of sacculi from BW25113 with lysozyme (upper panel) or lysozyme plus MepM (lower panel). The observed and calculated monoisotopic mass is indicated in Dalton. Peak a corresponds to a disaccharide-tripeptide substituted by a Lys-Arg (KR) dipeptide originating from digestion of the covalently-bond Braun lipoprotein by trypsin (Magnet *et al*, 2008).

B Discrimination of isomers containing 3→3 (Tri-Tetra) and 4→3 (Tetra-Tri) cross-links by tandem mass spectrometry. All fragments lost both GlcNAc molecules. Fragments that are specific of each isomer are shown in red. Fragments specific of an isomer but which can also be found in the other isomer following loss of a water molecule are shown in blue. Mass of fragments is shown in Dalton. A, L-Ala or D-Ala; a, C-terminal D-Ala; E, D-Glu; DAP, diaminopimelic acid.

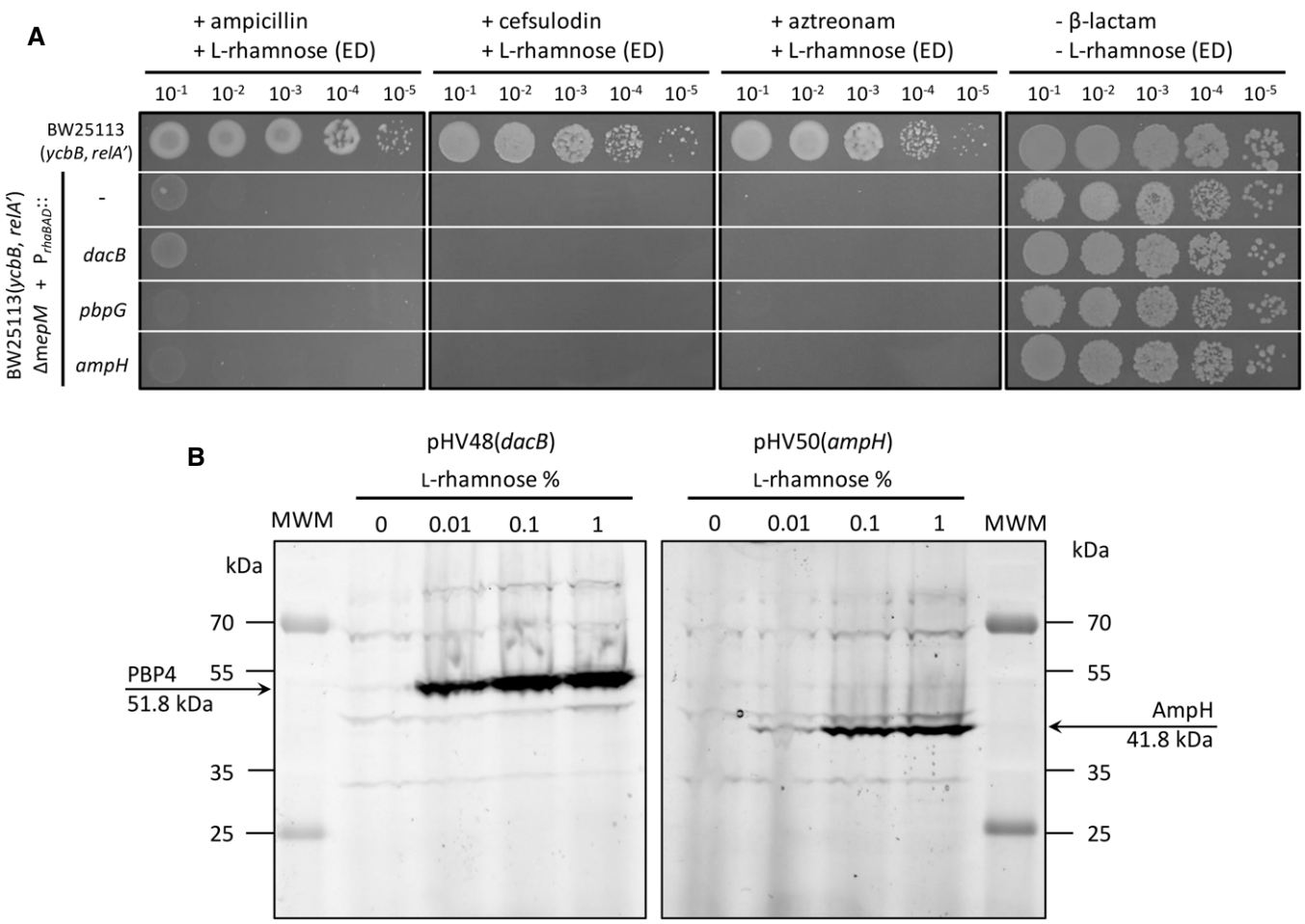

Figure EV3. Complementation of the *mepM* deletion by endopeptidases of the PBP family.

A Functional complementation of the *mepM* deletion of BW25113(*ycbB*, *relA*′) Δ*mepM* was performed with the pHV30 vector or recombinant plasmids encoding PBP4, PBP7, and AmpH under the control of the P_{rhaBAD} promoter. Induction of endopeptidase (ED) genes was performed with 0.2% L-rhamnose in the presence or absence of 16 μg/ml ampicillin, 32 μg/ml cefsulodin, or 8 μg/ml aztreonam. BHI agar plates contained 40 μM IPTG and 1% L-arabinose for induction *ycbB* and *relA*′, respectively. It was previously shown that ampicillin, cefsulodin, and aztreonam do not inhibit peptidoglycan cross-linking in BW25113(*ycbB*, *relA*′) due to full bypass of the D,D-transpeptidase activity of PBPs by the L,D-transpeptidase activity of YcbB, which is not inactivated by these drugs (Hugonnet *et al*, 2016).

B Control experiment showing that the P_{rhaBAD} promoter in plasmids pHV48(*dacB*) and phV50(*ampH*) enables inducible production PBP4 and AmpH. PBPs were labeled with a fluorescent β-lactam (BOCILLIN® FL Penicillin) in intact cells and separated by SDS–PAGE. This control experiment was only performed for PBP4 and AmpH since modifications of the resistance or growth phenotypes were observed for the induction of all other endopeptidase genes in at least one of the assays reported in the entire study. MWM, molecular weight marker.

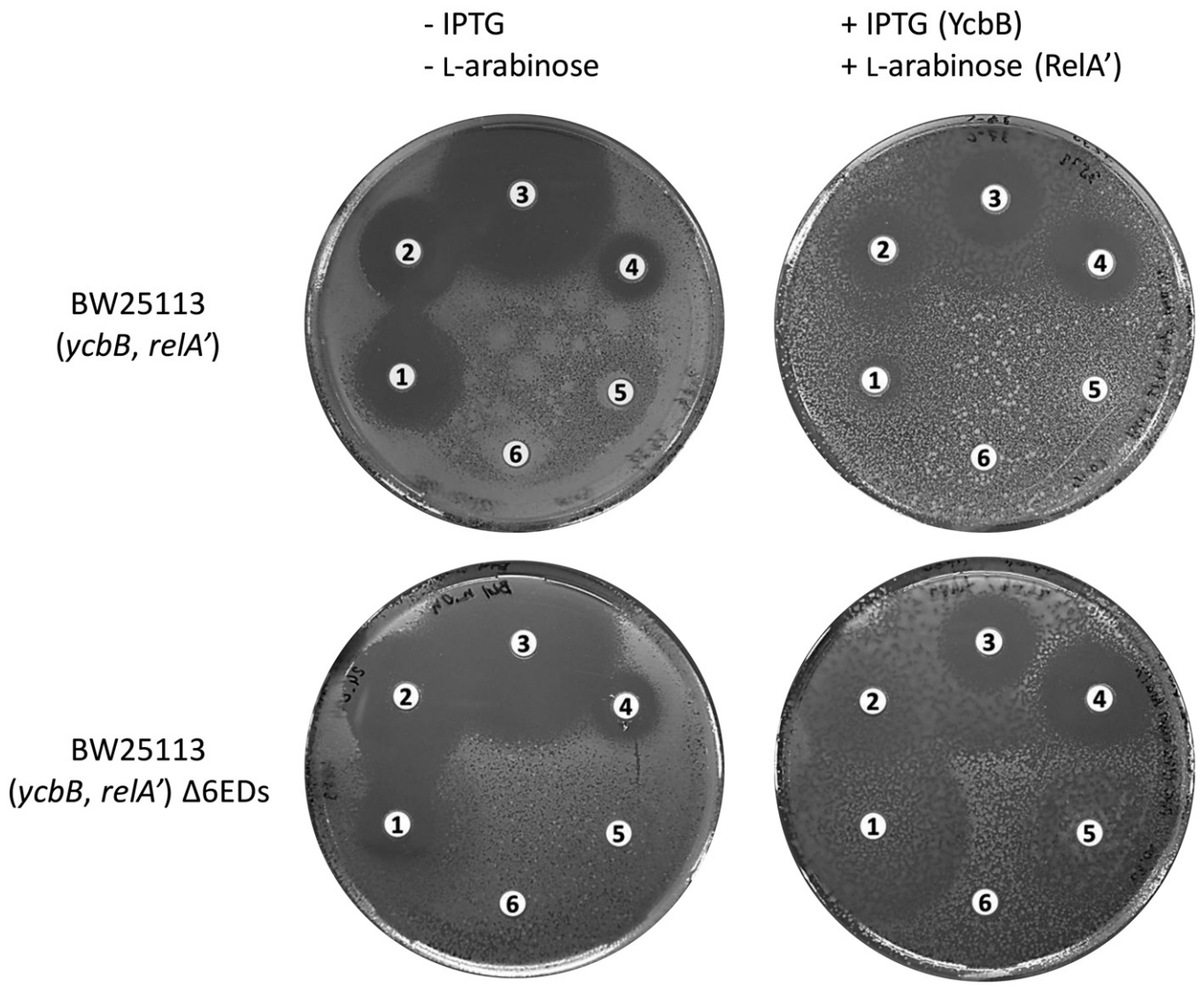

Figure EV4. Antibiotic susceptibility testing by the disk diffusion assay.

Disks contained 10 μg mecillinam (1), 10 μg ampicillin (2), 30 μg ceftriaxone (3), 30 μg tetracycline (4), 30 μg chloramphenicol (5), and 30 μg kanamycin (6). Plasmids pKT2 (*ycbB*) and pKT8(*relA'*) confer resistance to tetracycline and chloramphenicol, respectively. Kanamycin resistance is mediated by the Km^R cassette inserted in place of *relA*. Induction of *ycbB* and *relA'* was performed with 40 μM IPTG and 1% L-arabinose.

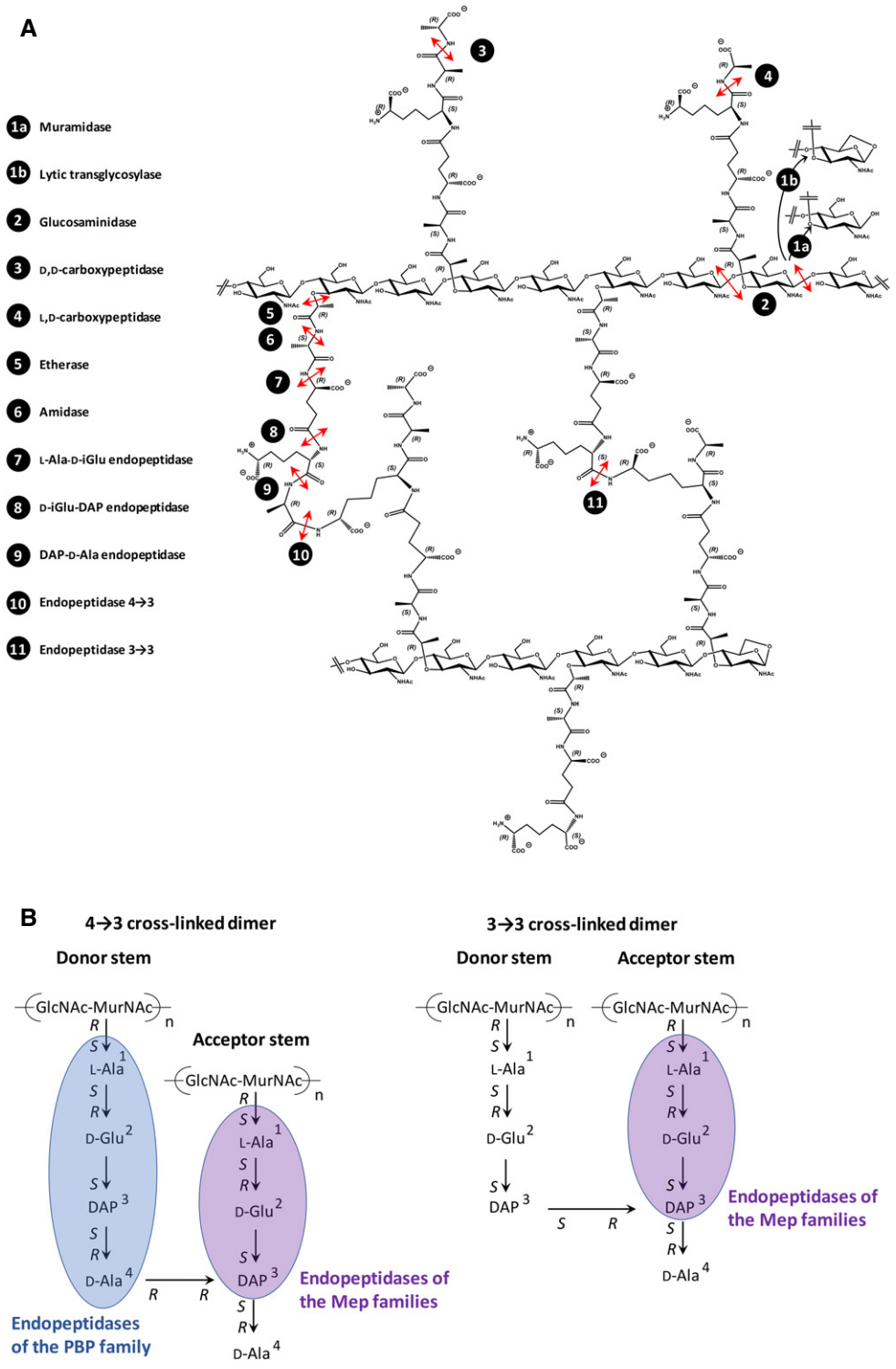

Figure EV5. Specificity of PG hydrolases.

A Highlight of enzyme stereospecificity. The commonly used endopeptidase designation was employed in the entire manuscript to refer to the cleavage of internal bonds although certain enzymes do not cleave peptide bonds connecting the α amino and carboxyl groups of two consecutive amino acids and should have been more precisely referred to as amidases.

B Recognition of the donor and acceptor stems of dimers by PBP and Mep endopeptidases accounting for the 4→3 versus 4→3 plus 3→3 specificities. According to this model, endopeptidases of the Mep families cleave both 4→3 plus 3→3 cross-links since they interact with the tripeptide portion of the acceptor stem, which is present in both types of dimers. In contrast, endopeptidases of the PBP family specifically interact with the tetrapeptide donor stem of 4→3 cross-linked dimers.