doi:10.1016/j.jmb.2010.03.033 J. Mol. Biol. (2010) 398, 507–517

JMB

Available online at www.sciencedirect.com

ScienceDirect

ELSEVIER

# Cleavage Specificity of *Enterococcus faecalis* EnpA (EF1473), a Peptidoglycan Endopeptidase Related to the LytM/Lysostaphin Family of Metallopeptidases

**François Reste de Roca[1,2,3], Caroline Duché[1,2,3], Shengli Dong[4], Alain Rincé[5], Lionel Dubost[6,7], David G. Pritchard[4], John R. Baker[4], Michel Arthur[1,2,3] and Stéphane Mesnage[1,2,3]***

[1]*Centre de Recherche des Cordeliers, LRMA, INSERM U872, Paris, France*

[2]*Université Pierre et Marie Curie, UMR-S 872, Paris, France*

[3]*Université Paris Descartes, UMR-S 872, Paris, France*

[4]*Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

[5]*Laboratoire de Microbiologie de l'Environnement, Institut de Biologie Fondamentale et Appliquée, Université de Caen, INRA, USC 2017-EA 956, Caen, France*

[6]*Muséum National d'Histoire Naturelle, USM0502, Paris, France*

[7]*Plateforme de Spectrométrie de Masse et de Protéomique du Muséum, Département de Recherche Développement et Diversité Moléculaire, CNRS, UMR8041, Paris, France*

*Enterococcus faecalis* EnpA (EF1473) is a 1721-residue predicted protein encoded by prophage 03 that displays similarity to the staphylolytic glycyl–glycyl endopeptidases lysostaphin and LytM. We purified a catalytically active fragment of the protein, $EnpA_C$, comprising residues 1374–1505 and showed that the recombinant polypeptide efficiently cleaved cross-linked muropeptides generated by muramidases, but was poorly active in intact sacculi. Analysis of the products of digestion of purified dimers by mass spectrometry indicated that $EnpA_C$ cleaves the D-Ala-L-Ala bond formed by the D,D-transpeptidase activity of penicillin-binding proteins in the last cross-linking step of peptidoglycan synthesis. Synthetic D was identified as the minimum substrate of $EnpA_C$ indicating that interaction of the enzyme with the donor peptide stem of cross-linked dimers is sufficient for its activity. Peptidoglycan was purified from various bacterial species and digested with mutanolysin and $EnpA_C$ to assess enzyme specificity. $EnpA_C$ did not cleave direct cross-links, but tolerated extensive variation in cross-bridges with respect to both their length (one to five residues) and their amino acid sequence. Recognition of the donor stem of cross-linked dimers could account for the substrate specificity of $EnpA_C$, which is significantly broader in comparison to endopeptidases belonging to the lysostaphin family.



**Corresponding author*. Centre de Recherche des Cordeliers, LRMA, INSERM U872, Paris, France. E-mail address: smesnage@hotmail.com.

Abbreviations used: PGN, peptidoglycan; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; PBP, penicillin-binding protein; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RP, reverse phase; DAP, diaminopimelic acid.

0022-2836/$ - see front matter 

*Received 22 February 2010;*
*received in revised form*
*17 March 2010;*
*accepted 18 March 2010*
Available online
27 March 2010

***Edited by R. Huber***

*Keywords:* autolysin; lysostaphin; endopeptidase; peptidoglycan cross-bridge; *Enterococcus faecalis*

## Introduction

Bacterial cell integrity and shape are ensured by peptidoglycan (PGN), the major component of the cell wall.[1,2] PGN is a gigantic bag-shaped molecule composed of glycan strands alternating β-1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, cross-linked by short peptides stems.[3] Peptide stems are bound to the D-lactoyl residue of MurNAc through their N-terminus and contain amino acids with L and D configurations (Fig. 1). The peptide stem is usually made of L-alanine, D-glutamic acid linked by its γ/carboxyl group to the L center of the following residue, a diamino acid, and two D-alanines.[4] In Gram-positive bacteria, the α-carboxyl of D-Glu is generally amidated (D-iGln). The pentapeptide stem (L-Ala-D-iGln-L-Lys-D-Ala-D-Ala) is conserved in pathogens belonging to the genera *Enterococcus*, *Streptococcus*, and *Staphylococcus*. The ε/amino group of L-Lys is substituted by a side chain of variable lengths and compositions:[4] two L-Ala in *Enterococcus faecalis*,[5] five Gly in *Staphylococcus aureus*,[6] the sequence L-Ala-L-Ser or L-Ala-L-Ser-L-Ala in *Weissella viridescens*,[7] and D-Asn or D-Asp in *Enterococcus faecium*[8] and *Lactococcus lactis*.[9] L-Amino acids and glycine are activated as aminoacyl tRNAs and transferred to precursors by a family of nonribosomal peptide-bond-forming enzymes (Fem enzymes).[10] Incorporation of D-amino acids into PGN precursors involves a distinct class of enzymes that use aminoacyl phosphates as substrates. Branched PGN precursors are polymerized by D,D-transpeptidase activity of multimodular penicillin-binding proteins (PBPs),[11] which link the carbonyl of D-Ala$_4$ of a peptide stem (acyl donor) to the N-terminal residue of the side chain (acyl acceptor), thereby forming PGN cross-bridges (Fig. 1).

During growth, the PGN network constantly undergoes partial hydrolysis for incorporation of PGN precursors, septum cleavage after division, or assembly of macromolecular structures such as secretion apparatus or flagella.[12] Genome analyses revealed that most bacteria contain a rather large

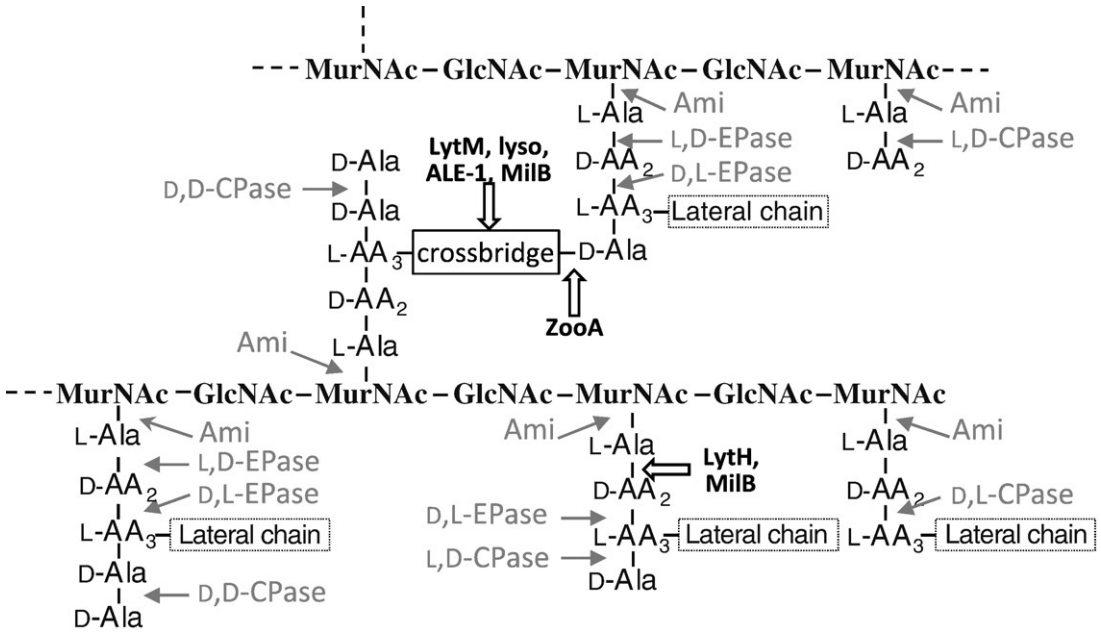


**Fig. 1.** Site of action of PGN amidases. *N*-Acetylmuramyl-L-alanine amidases (Ami) hydrolyze the amide bonds between the lactyl group of MurNAc and the L-alanine of the stem peptide. Endopeptidases (L,D-EPase and D,L-EPase) cleave amide bonds in the peptides. Carboxypeptidases (D,D-CPase, L,D-CPase, and D,L-CPase) hydrolyze peptide bonds to remove C-terminal D-amino acids or L-amino acids. Three major variations found in Gram-positive PGN are shown: L-amino acid in position 2 ($AA_2$) can be either D-iGlu or D-iGln; L-diamino acid in position 3 (AA3) is most often L-Lys, but can also be *meso*-DAP or its amidated derivative; and PGN cross-bridges have variable lengths and compositions.