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Research paper

# Unusual substrate specificity of the peptidoglycan MurE ligase from *Erysipelothrix rhusiopathiae*

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ABSTRACT

*Erysipelothrix rhusiopathiae* is a Gram-positive bacterium pathogenic to many species of birds and mammals, including humans. The main feature of its peptidoglycan is the presence of L-alanine at position 3 of the peptide stem. In the present work, we cloned the *murE* gene from *E. rhusiopathiae* and purified the corresponding protein as $His_6$-tagged form. Enzymatic assays showed that *E. rhusiopathiae* MurE was indeed an L-alanine-adding enzyme. Surprisingly, it was also able, although to a lesser extent, to add *meso*-diaminopimelic acid, the amino acid found at position 3 in many Gram-negative bacteria, Bacilli and Mycobacteria. Sequence alignment of MurE enzymes from *E. rhusiopathiae* and *Escherichia coli* revealed that the DNPR motif that is characteristic of *meso*-diaminopimelate-adding enzymes was replaced by HDNR. The role of the latter motif in the interaction with L-alanine and *meso*-diaminopimelic acid was demonstrated by site-directed mutagenesis experiments and the construction of a homology model. The overexpression of the *E. rhusiopathiae murE* gene in *E. coli* resulted in the incorporation of L-alanine at position 3 of the peptide part of peptidoglycan.



## 1. Introduction

*Erysipelothrix rhusiopathiae* is a small, non motile, non-sporulating, Gram-positive rod bacterium which possesses a capsule [1]. This organism, which is ubiquitous in nature, is pathogenic or commensal in a wide variety of animals [2]. *E. rhusiopathiae* is an opportunistic pathogen infecting many species of mammals, including humans [3]. It is responsible for swine erysipelas, a disease of great prevalence and economic importance [4]. In humans, three clinical forms exist: a localised cutaneous form, erysipeloid; a generalised cutaneous form; and a septicaemic form which is often associated with endocarditis [5]. The genome of *E. rhusiopathiae* has been completely sequenced and has been shown to be similar to those of other Gram-positive bacteria. It possesses in particular a full set of peptidoglycan biosynthesis genes, which are relatively dispersed throughout the genome. However, it is not clear whether all the genes involved in the complete biosynthetic pathways of wall teichoic acids and lipoteichoic acids are present [6].

Peptidoglycan (murein) is the rigid macromolecule that surrounds and protects bacterial cells, preserving membrane integrity by withstanding the inner osmotic pressure. It is composed of glycan chains made of alternating *N*-acetylglucosamine and *N*-acetylmuramyl residues that are cross-linked by short peptides bridges [7,8]. Different compositions have been published for the peptidoglycan of *E. rhusiopathiae* [9–11]. In 2001, Schubert and Fiedler have established its correct structure (Fig. 1). It possesses L-serine at position 1 and L-alanine at position 3 of the peptide stem. Cross-linkage extends between D-glutamic acid at position 2 of one peptide subunit and D-alanine at position 4 of another via a glycine-L-lysine-L-lysine interpeptide bridge [12].

Because peptidoglycan is unique to bacteria, the enzymes that

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**Abbreviations**

| | |
|---|---|
| $A_2pm$ | 2,6-diaminopimelic acid |
| ESI | electrospray ionization |
| HPLC | high-performance liquid chromatography |
| IPTG | isopropyl β-D-thiogalactopyranoside |
| MALDI-TOF | matrix-assisted laser desorption/ionization mass spectrometry time-of-flight |
| MurNAc | N-acetylmuramic acid |
| $Ni^{2+}$-NTA | $Ni^{2+}$-nitrilotriacetate |
| PBP | penicillin-binding protein |
| PCR | polymerase chain reaction |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |

*Subscripts*

| | |
|---|---|
| Er | *Erysipelothrix rhusiopathiae* origin of genes and enzymes |
| Ec | *Escherichia coli* origin of genes and enzymes |
| Sa | *Staphylococcus aureus* origin of genes and enzymes |

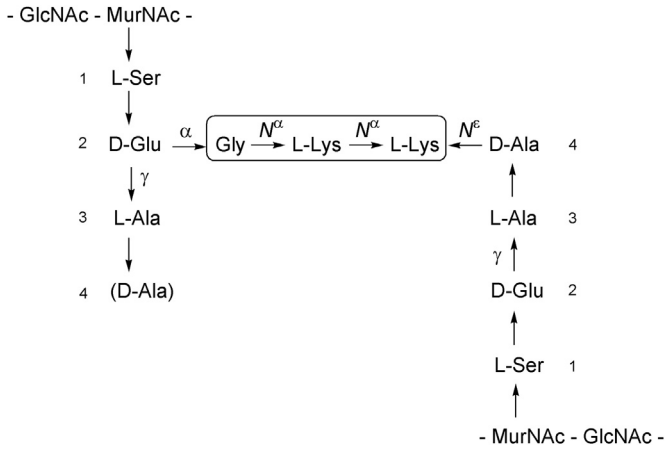


**Fig. 1.** Schematic representation of the peptidoglycan from *E. rhusiopathiae*. The interpeptide bridge is boxed. Since the molar ratio of L-Ala to D-Ala in final peptidoglycan is 2:1, the terminal D-Ala residue (in parentheses) is probably missing. Adapted from Ref. [12].

synthesize it are very interesting targets for antibiotic therapy. The cytoplasmic synthesis of the peptide part of the monomer unit is ensured by a family of enzymes called the Mur ligases [13–15]. These ligases (MurC, MurD, MurE and MurF) share common structural and mechanistic features [16–19]. Among them, MurE catalyzes the addition of the third amino acid residue. This residue, generally a diamino acid, varies among the bacterial species: it is *meso*-diaminopimelic acid (*meso*-$A_2pm$) for most Gram-negative bacteria, Mycobacteria and Bacilli, L-lysine for most Gram-positive bacteria (Firmicutes), L-ornithine for Spirochetes; in rare cases, other amino acids (*meso*-lanthionine, LL-$A_2pm$, L-diaminobutyric acid, L-homoserine, L-glutamic acid, L-alanine) [8,11,13] are found. Amino acid at position 3 is often involved in the cross-linking. So MurE ligase plays a crucial role in peptidoglycan biosynthesis: it must be highly specific for the relevant amino acid in order to avoid the incorporation of an incorrect amino acid which could result in cell lysis [20]. The crystallization of the MurE enzymes from *Escherichia coli* ($MurE_{Ec}$) and *Staphylococcus aureus* ($MurE_{Sa}$) has allowed to decipher the structural bases of this high specificity [21,22]. In particular, from sequence alignments of different MurE orthologues, two short specific consensus motifs were identified in the amino acid-binding site: DNPR for *meso*-$A_2pm$ recognition and D(D/N)P(N/A) for L-Lys recognition [22–24].

For *E. rhusiopathiae* MurE ($MurE_{Er}$), the situation may be different since the amino acid at position 3 does not participate in the cross-linking. Interestingly, $MurE_{Er}$ does not possess any of the two latter consensus motifs. In this paper, we report on the overproduction and purification of $MurE_{Er}$, as well as the characterization of its properties and substrate specificity. The effects of overexpressing this gene on the peptidoglycan composition and structure of *E. coli* are also analyzed.

## 2. Materials and methods

### 2.1. Materials

DNA restriction enzymes and synthetic oligonucleotides were purchased from New England Biolabs and Eurofins-MWG, respectively. UDP-MurNAc-L-Ala-D-Glu, UDP-MurNAc-Gly-D-Glu, UDP-MurNAc-L-Ser-D-Glu and UDP-MurNAc-L-Ala-D-[$^{14}$C]Glu were prepared according to published procedures [25–27]. The enzymatic syntheses of UDP-MurNAc-[$^{14}$C]Gly-D-Glu, UDP-MurNAc-L-[$^{14}$C]Ser-D-Glu and UDP-MurNAc-L-Ala-γ-D-Glu-L-Ala are described in the Supplementary Material.

### 2.2. General DNA techniques and E. coli cell transformation

The constructions of the pET2160 and p*Trc*His60 plasmid vectors have been previously described [28,29]. Plasmid purification kits were purchased from Macherey–Nagel. Standard procedures for molecular biology were used [30]. *E. coli* cells were made competent and transformed with plasmid DNA according to Dagert and Ehrlich [31], or by electroporation.

### 2.3. Bacterial strains and growth conditions

*E. coli* strains DH5α (Invitrogen), BL21(DE3)/pLysS (Novagen) and TKL-11 [32,33] were used as hosts for plasmids, overproduction of the $MurE_{Er}$ enzyme and complementation assays, respectively. DH5α and BL21(DE3)/pLysS were grown in 2YT medium [34], and TKL-11 was grown in low salt LB medium (0.2% NaCl instead of 0.5% NaCl) supplemented with thymine (100 $\mu g \cdot ml^{-1}$). Growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. When required, ampicillin and chloramphenicol were added at concentrations of 100 and 25 $\mu g \cdot ml^{-1}$, respectively.

### 2.4. Construction of plasmids

The *E. rhusiopathiae murE* gene was amplified from the LOG 95 serotype in order to be cloned into the pET2160 plasmid. Because of the existence of an internal BspHI restriction site in the $murE_{Er}$ gene, the procedure needed a two-step PCR protocol. First, two PCR fragments were generated using two couples of primers. The couple 1 is 5′-GCGT**TCATGA**AATTGAATAAACTTGTGAACACA-GAATTGG-3' (underlined the start codon, in bold an engineered BspHI site) and 5′-GGATAGGTTTTTCCTTC**G**TGAATCAGTGTGAATTC GG-3' (in bold modified nucleotide abolishing the internal BspHI site without modifying the amino acid sequence); and the couple 2 is 5′-CCGAATTCACACTGATTCA**C**GAAGGAAAAACCTATCC-3' (in bold the complementary nucleotide to abolish the internal BspHI site) and 5′-CTAC**AGATCT**CTTAATTTCATTTTCTTTTCCTCCG-3' (in bold an engineered BglII site). Then, a second round of PCR was done with the two external primers and with the first purified products. The