Structural enzymology of bacterial cell wall biosynthesis: Cloning, Expression and Purification of *E. coli* MurC

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Introduction

Increasing prevalence of antibiotic resistance has fuelled research into novel targets for antibacterial drugs. Peptidoglycan (murein) is the principle constituent of the bacterial cell wall and is responsible for rigidity of the cell against internal osmotic pressure. Peptidoglycan is composed of glycan chains and peptides and steps involved in cross-linking the peptidoglycan chains are inhibited by the β-lactam antibiotics such as penicillin and cephalosporins. The biosynthesis of peptidoglycan is a multistep pathway of 11 steps, divided into 3 main phases[1], the second phase is of most interest here (***Figure-1***).

1. Formation of UDP-N-acetyl muramic acid (UDPMurNAc)
2. A pentapeptide chain is added sequentially to UDPMurNAc by four ATP-dependent amide bond ligases
3. Transport through the cytoplasm and incorporation into the growing polypeptide layer

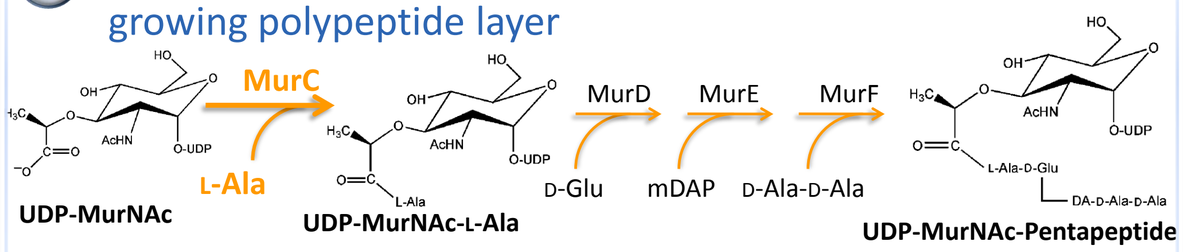

Figure-1 – The cytoplasmic steps of peptidoglycan biosynthesis. The four Mur ligases: MurC, D E and F catalyse the sequential addition of the pentapeptide side chain to the D-lacyl group of UDP-MurNAc[2].

Mur Ligases

MurC catalyses the addition of L-alanine to the lactyl group of UDPMurNAc[2]. A sequential, ordered kinetic mechanism has been demonstrated, with ATP binding first, UDP-MurNAc second and L-Ala third. The reaction is reversible and the exchange reaction is phosphate- and ADP-dependent[3]. The enzyme is composed of three structural domains (***Figure-2***). The active site of MurC lies at the junction of the three structural domains and comprises specific binding pockets for the three substrates: UDPMurNAc, MgATP and L-alanine[4].

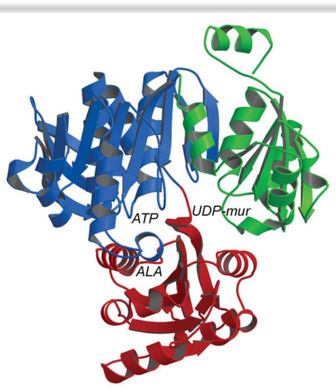

Figure-2 – Cartoon structure of *E. coli* Mur C, comprising an N-terminal domain that binds UDPMurNac, a central catalytic domain that resembles an ATP-binding domain and a C-terminal domain that binds to the incoming amino acid

Cloning *murC*

Polymerase Chain Reaction (PCR)

Primers specific the 5' and 3' ends of the *murC* gene were designed, to enable amplification of the gene by PCR (***Figure-3***). The restriction sites for *NdeI* and *XhoI* restriction endonucleases were included in the primers to enable unidirectional cloning into the destination vector after preparation with the same enzymes.

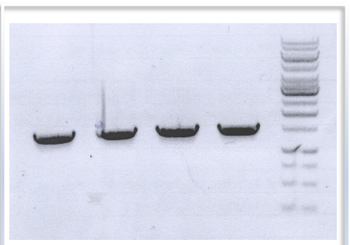

Figure-3 – PCR products: *murC*

Digestion

The *E. coli* plasmid vector pET22b was chosen as the vector for cloning *murC*. PCR products of the amplified *murC* gene and pET22b vector were digested with the restriction endonucleases *NdeI* and *Xho1* prior to ligation .

Figure-4 – pET22b plasmid vector and *murC*

Ligation

The *NdeI* and *XhoI* digested pET22b and *murC* PCR fragment (***Figure-4***) were ligated using T4 DNA ligase for 2 hours at room temperature.

Cloning continued...

Transformation

Competent Top10 *E. coli* cells were transformed with the ligation mixture, single colonies were selected, grown and analysed by restriction digest. Colonies 1, 3 and 6 contained the correct construct (***Figure-5*** and ***Figure-6***).

Digestion

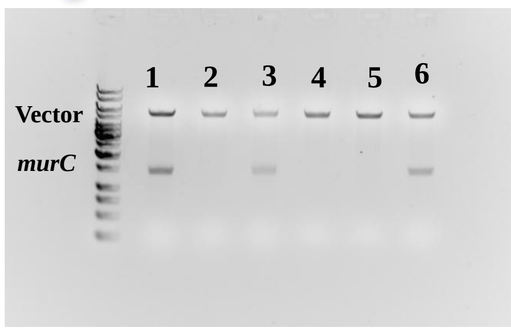

Figure-5 – The construct was digested with *XhoI* and *NdeI* to free the insert from the vector to confirm the construct had successfully inserted.

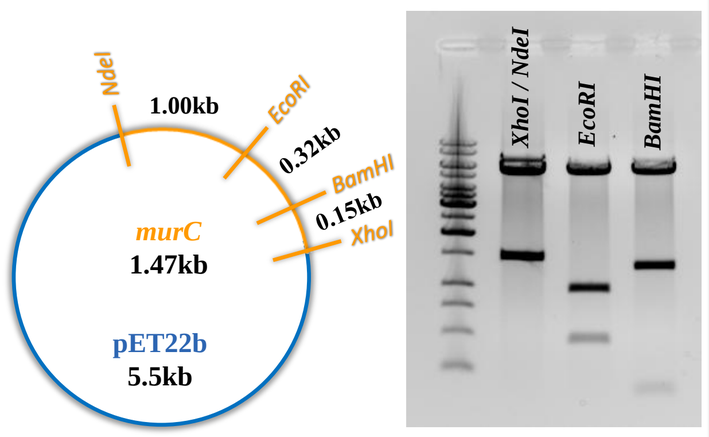

Figure-6 - The *murC* gene is known to contain the restriction sites for *EcoRI* and *BamHI* endonucleases. A digest was set up to cleave the insert at these restriction sites to confirm the insert was *murC*.

Expression of MurC

Transformation

MurC was overexpressed in *E. coli* B834(DE3) expression strain (***Figure-7***).

Induction

A small scale test culture was induced with isopropyl β-D-thiogalactopyranoside (ITPG) to ensure MurC expressed. A large 1L culture was then grown and induced with 0.1mM IPTG for 3 hours.

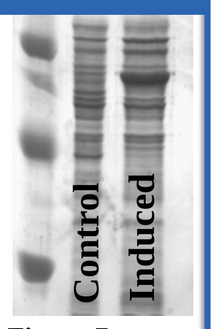

Figure-7 – Expression of MurC protein in the induced cells

Purification of MurC

IMAC (Immobilized Metal Affinity Chromatography)

A 6 His-tag was fused to the C-terminal of the MurC protein during cloning. The advantage of the His-tag is to allow rapid purification on an affinity nickel column. Nickel acts as a metal chelator to purify the protein, a method known as IMAC. The protein is eluted from the column at an optimum concentration of imidizole buffer.

Gel Filtration

MurC was further purified using a Superdex-200 preparative column (***Figure-8a***) and the results were analysed by SDS-PAGE (***Figure-8b***).

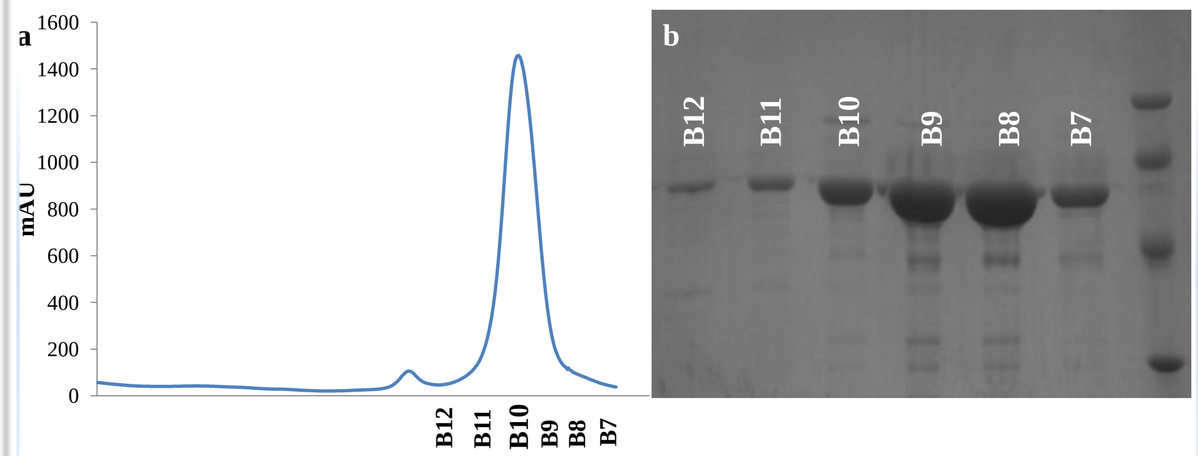

Figure-8 – MurC purification results: (**a**) Trace of fractions eluted from gel filtration (**b**) SDS-PAGE analysis of pure fractions

Conclusion

The *murC* gene was successfully cloned and its product expressed and purified. Crystallization trays have been set up to obtain crystals suitable for soaking experiments with the Pfizer ATP site directed inhibitor C1.

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References: [1] Deva, T., Pryor, K. D., Leiting, B., Baker, E. N., Smith, C. A. (2003) Purification, crystallization and preliminary X-ray analysis of *Escherichia coli* UDP-*N*-acetylmuramoyl:L-alanine ligase (MurC) *Acta Crystallogr D Biol Crystallogr* **59**: 1510-1513 [2] Smith, C. A. (2006) Structure, Function and Dynamics in the *mur* Family of Bacterial Cell Wall Ligases *J. Mol. Biol.* **362**: 640-655 [3] Barreteau, H., Kovac, A., Boniface, A., Sova, M., Gobec, S. and Blanot, D. (2008) Cytoplasmic steps of peptidoglycan biosynthesis *FEMS Microbiol Rev* **32**: 168-207 [4] Deva, T., Baker, E. N., Squire, C. J. and Smith, A. C. (2006) Structure of *Escherichia coli* UDP-*N*-acetylmuramoyl:L-alanine ligase (MurC) *Acta Crystallogr D Biol Crystallogr* **62**: 1466-1474