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Cloning, expression and rapid purification of active recombinant mycothiol ligase as B1 immunoglobulin binding domain of streptococcal protein G, glutathione-*S*-transferase and maltose binding protein fusion proteins in *Mycobacterium smegmatis*

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Abstract

Mycothiol ligase (MshC) is a key enzyme in the biosynthesis of mycothiol, a small molecular weight thiol found in *Mycobacteria* spp. and other actinomycetes. Mycothiol plays a fundamental role in these organisms by helping to provide protection from the effects of reactive oxygen species and electrophiles, including many antibiotics. It has recently been demonstrated that the MshC gene and more generally the production of mycothiol are essential to *Mycobacterium tuberculosis*, indicating that MshC may represent a novel target for new classes of antituberculars. Because MshC cannot be expressed heterologously in *Escherichia coli* and isolation from *Mycobacterium smegmatis* is impractical, we have optimized the *E. coli–M. smegmatis* shuttle vector pACE for cloning and recombinant expression of MshC (under control of an acetamidase-inducible promoter). To improve expression levels and simplify purification, we further constructed three N-terminal-MshC fusion proteins where N-terminal tags included the B1 domain of streptococcal protein G (to give GB1-MshC), glutathione-*S*-transferase (to give GST-MshC) and maltose binding protein (to give MBP-MshC), for expression in *M. smegmatis*. By expressing all three fusion proteins in a mutant strain of *M. smegmatis* mc^2155, namely I64 L205P MshC *M. smegmatis* which lacks mycothiol ligase activity, we demonstrate in vivo mycothiol ligase activity for each construct. Recombinant GST-MshC and MBP-MshC were isolated in one step by affinity chromatography in a yield of 0.7 and 1.2 mg fusion protein/L and exhibited specific activities of 9 nmol min^{-1} mg^{-1} and 25 nmol min^{-1} mg^{-1}, respectively.

Keywords: MshC; Mycothiol; Fusion proteins; Affinity purification; Mycothiol ligase; Complementation; *Mycobacterium tuberculosis*; Shuttle vector

Introduction

Mycothiol (MSH, AcCys-GlcN-Ins)[1] is the dominant low molecular weight thiol that is so far unique to actinomycetes [1,2] and has been shown to be present in all major groups of actinomycetes [1]. Mycothiol comprises one unit each of *N*-acetylcysteine (AcCys), 1-D-*myo*-inositol (Ins) and 2-amino-2-deoxy-D-glucopyranose (GlcN). Mycothiol is

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[1] *Abbreviations used:* MSH, AcCys-GlcN-Ins, 1-D-*myo*-inosityl-2-(*N*-acetyl-L-cysteinyl) amido-2-deoxy-α-D-glucopyranoside; GlcN-Ins, 1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyrano-side; Cys-GlcN-Ins, 1-D-*myo*-inosityl-2-(L-cysteinyl) amido-2-deoxy-D-glucopyranoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GB1, B1 immunoglobulin binding domain of streptococcal protein G; GST, glutathione-*S*-transferase; MBP, maltose binding protein; DTT, dithiothreitol; SDM, site directed mutagenesis; DAD, diode array detector; API-ES, atmospheric pressure electrospray ionization; MSD, mass selective detector; ORF, open reading frame.

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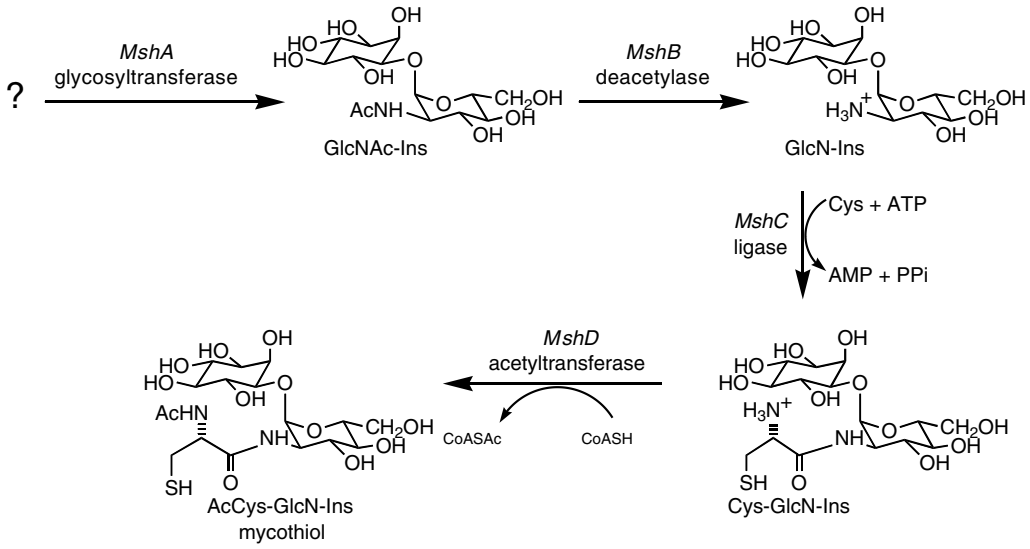

Fig. 1. Biosynthetic scheme of mycothiol.

assumed to be the functional equivalent of glutathione in mycobacteria and has been shown to be involved in protecting *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* from toxic oxidants and antibiotics [3,4]. Owing to the demonstration that mycothiol-deficient mutants become hypersensitive to some first line antituberculars, interest in identifying inhibitors of mycothiol biosynthesis and mycothiol-assisted detoxification has continued to rise [5].

Mycothiol biosynthesis occurs in at least four steps and involves the gene products of *mshA* through *mshD*. The third committed step in mycothiol biosynthesis involves transfer of L-cysteine (Cys) to GlcN-Ins to produce Cys-GlcN-Ins and is catalyzed by the enzyme mycothiol ligase, or *mshC* (L-Cys:1D-*myo*-inosityl 2-amino-2-deoxy-α-D-glucopyranoside ligase), in an ATP-dependent manner [2,6] (Fig. 1). Recently, the *mshC* gene has been shown to be essential for growth in *M. tuberculosis* by targeted gene disruption [7] and by transposon site hybridization [8]. When a second copy of *mshC* was inserted into the genome prior to targeted inactivation of the native *mshC* gene, viable mutants were isolated [7]. Since MshC has been shown to be required for MSH biosynthesis [4], these studies indicate that mycothiol is essential for growth in *M. tuberculosis* strains provoking further study of this enzyme as a therapeutic target.

The initial report describing the identification and characterization of MshC relied on laborious isolation methods from large scale cultures of *M. smegmatis* that were further hampered by the production of two MshC protein products that differed by addition of eight amino acids on the N-terminus [6]. In contrast to heterologous production of most proteins that can be expressed well in *Escherichia coli*, there is ample evidence that the proteins derived from *M. tuberculosis* are refractory to these conditions [9–12]. Because a prerequisite for screening small molecule libraries to identify inhibitors of MshC will rely on reproducible production of relatively large amounts of active enzyme, we have optimized the *E. coli*-mycobacterial shuttle vector pACE [13,14] to provide a robust cloning and expression vector system for production of MshC using *M. smegmatis* as the expression host. Fusion to three unrelated proteins through the N-terminus of MshC increases production and greatly facilitates purification. We believe these expression vectors will be of general value for production of proteins derived from *M. tuberculosis.*

Materials and methods

Construction of mshC-tagged expression plasmids pACE/GB1-MshC, pACE/GST-MshC and pACE/MBP-MshC

The MshC gene was previously amplified from total genomic DNA of *M. tuberculosis* strain H37Rv and cloned into the *Bam*HI/*Hin*dIII sites of pRSETA (Invitrogen) [6]. The resulting plasmid pRSETA/MshC was used as a template for PCR amplification of MshC using *Taq* PCR master mix kit according to the manufacturer's instructions (Qiagen) together with an appropriate pair of forward and reverse primers designed for each of three fusion protein constructs. The primer sequences used are shown in Table 1 and numbered 1, 2 and 4. The amplified MshC gene products were subcloned into the *Bam*HI/*Xho*I sites of pGEV2 [15], *Nco*I/*Bam*HI of pET-42a (Novagen) or *Bam*HI/*Hin*dIII sites of pMALc2X (New England Biolabs) using a quick ligation kit (New England Biolabs) to generate constructs encoding GB1, GST-H_6-S and MBP fusion proteins, respectively. Note that insertion of the MshC gene into the *Nco*I/*Bam*HI sites of pET-42a generates a fusion construct in which GST and MshC are separated by a 6-His and S tag-linker, referred to above as GST-H_6-S and as GST from here on. The corresponding constructs, referred to as pGEV/MshC (for expression of GB1-MshC), pET42a/MshC-2 (for expression of GST-MshC after frame correction of pET42a/MshC by primer set 3 listed in Table 1) and pMALc2X/MshC (for expression of MBP-MshC), were subsequently used as tem-