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Purification and characterization of recombinant malate synthase enzymes from *Streptomyces coelicolor* A3(2) and *S. clavuligerus* NRRL3585

P Loke[a], L-L Goh, B Seng Soh, P Yeow and T-S Sim

Department of Microbiology, Faculty of Medicine, National University of Singapore, MD4A, 5 Science Drive 2, Singapore 117597, Singapore

Malate synthases (MS) from *Streptomyces coelicolor* A3(2) and *S. clavuligerus* NRRL3585 were cloned by polymerase chain reaction into a glutathione *S*-transferase (GST) fusion expression vector and heterologously expressed in *Escherichia coli*. The fusion GST–MS construct improved the soluble expression of MS by approximately 10-fold compared to the soluble expression of nonfusion MS. With the significant improvement in levels of soluble MS, purification and subsequent cleavage of recombinant MS from GST were facilitated in this study. Using purified enzymes, optimized parameters, which achieved maximal specific activity, were established in the enzymatic assay for streptomycete MS. The average purified specific activities of *S. coelicolor* and *S. clavuligerus* MS were 26199 and 11821 nmol/mg min, respectively. Furthermore, enzymatic analysis revealed that the two streptomycete MS displayed a similar K_m value for acetyl-CoA, but *S. coelicolor* MS had a K_m value for glyoxylate that is approximately sixfold higher than *S. clavuligerus* MS.
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Introduction

Malate synthase (MS) is a pivotal enzyme in the glyoxylate bypass of microorganisms, fungi and plants. It catalyzes the aldol condensation of glyoxylate and acetyl-CoA to give malate, which returns to the tricarboxylic acid cycle, thus replenishing key cellular intermediates [12]. The roles of MS are rather diverse as it has been implicated in glyoxysomes of plants for seedling germination, in starved cotyledons by providing four-carbon acids for respiration, in the utilization of stored fat during hibernation in black bears, in the biodegradation of high-molecular-weight polyethylene glycols in *Pseudomonas stutzeri* and in allantoin metabolism in *Saccharomyces cerevisiae* [3,7,11,17,22].

A number of MS genes from different organisms, ranging from bacteria and fungi to plants, have been cloned and studied although no MS gene sequence of animal origin has been obtained. In *Streptomyces* spp., three MS have been cloned, of which two of them, i.e., from *Streptomyces clavuligerus* and *S. coelicolor*, have been heterologously expressed in *Escherichia coli* [2,14]. Furthermore, positive signals were detected from the genomic DNA of 15 *Streptomyces* spp. hybridized with a probe based on the *S. arenae* MS gene, suggesting that most streptomycetes carry this gene [9].

However, the purification and enzymatic studies of recombinant MS from *Streptomyces* spp. have not been reported, prompting us to investigate the enzymatic properties of streptomycete MS. To this end, we have employed polymerase chain reaction (PCR) to clone the MS gene from *S. clavuligerus* and *S. coelicolor* into a glutathione *S*-transferase (GST) fusion vector, pGEX-6P-1 (Amersham Pharmacia Biotech, Singapore) for ease of expression and purification. Intriguingly, we found that the fusion of MS to GST improved the expression of MS in the soluble form as compared to the nonfusion protein.

Materials and methods

Growth of cultures and genomic DNA extraction

S. coelicolor A3(2) strain M130 and *S. clavuligerus* NRRL3585 were grown in tryptone soya broth (Oxoid, Singapore) at 28°C using a rotary shaker, whereas *E. coli* strains were cultured in Luria–Bertani medium [20] at 37°C. The relevant antibiotics were added at a final concentration of 50 μg/ml when the *E. coli* strain harbors a resistance plasmid. Extraction of genomic DNA from *Streptomyces* spp. was performed using the method of Hopwood *et al* [8].

Primer design, cloning and DNA sequencing

The cloning primers were designed based on the *S. coelicolor* and *S. clavuligerus* MS gene (*aceB*) sequences previously published from our laboratory and deposited under the GenBank accession numbers AF206498 and AF070989, respectively [2,14] (Table 1). The restriction enzyme sites for *Bam*HI and *Eco*RI were incorporated into the forward and reverse primers, respectively, to facilitate subsequent subcloning into the GST fusion vector. PCR cloning was performed by employing the touchdown amplification profile of eight cycles of 95°C (45 s), 64–60°C (30 s), 72°C (2 min), followed by 27 cycles of 95°C (45 s), 64°C (30 s), 72°C (2 min) and a final extension step at 72°C for 7 min. Negative

Correspondence: Dr T-S Sim, Department of Microbiology, Faculty of Medicine, National University of Singapore, MD4A, 5 Science Drive 2, Singapore 117597, Singapore

[a]Current address: Laboratory of Cell and Molecular Biology, National Heart Centre, Mistri Wing, 17 Third Hospital Avenue, Singapore 168752, Singapore.
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Table 1 Cloning primers for *aceB* genes in this study

Purpose	Primer sequence (restriction enzyme sites in bold)[a]
Cloning of *S. coelicolor aceB*	Forward (OL417) 5′ **GGATCC**ATGTCCGCACCAGCGCCGTCCACG 3′ and reverse (OL281) 5′GAGC**GAATTC**GGCGTCAGCC CTGAGCTGC 3′
Cloning of *S. clavuligerus aceB*	Forward (OL189) 5′ **GGATCC**ATGTCCGCAGCCCCGTCC 3′ and reverse (OL190) 5′ T**GAATTC**TCAGCCGGTCAGCCGCTCGTA 3′

[a]The *Bam*HI and *Eco*RI restriction sites are incorporated into the forward and reverse primers, respectively, to facilitate cloning into the pGEX-6P-1 expression vector.

controls without the presence of genomic DNA were also run. The amplified blunt-end *aceB* PCR product was cloned into the cloning vector, pCR® -BluntII-TOPO, according to the manufacturer's instructions (Invitrogen, Singapore), and transformed into *E. coli* TOP10. Recombinant *aceB* constructs were extracted from overnight cultures using the Wizard® *Plus* SV Minipreps DNA Purification System (Promega, Singapore) and the *aceB* gene of interest was sequenced twice on both strands to confirm the exact identity of the gene sequence.

Heterologous expression and purification

Utilizing the relevant restriction enzymes, the *aceB* gene from recombinant pCR® -BluntII-TOPO constructs was digested and subsequently subcloned into pGEX-6P-1 for heterologous expression. Expression was carried out at 37°C until the OD_{600} of the *E. coli* BL21(DE3) cultures harboring the recombinant *aceB* fusion constructs reached 0.8–1.0, following which isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final of concentration of 1 mM. The cultures were further incubated at 20°C for 15 h for optimal expression of the cloned genes. The nonfusion *aceB* genes of *S. clavuligerus* and *S. coelicolor* previously cloned in pET24a vectors were used as controls [2,14].

Soluble cell-free extracts were obtained by sonication at an intensity of 15 μm for six cycles of 5-s pulses with a 55-s rest period between each burst, using the MSE Soniprep with a 1/8-in. probe. Before purification was performed, the cell debris was removed by centrifugation at 10,000 rpm for 15 min. The GST–MS fusion protein was purified by affinity chromatography using Glutathione Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer's instructions. An appropriate amount of PreScission™ Protease (Amersham Pharmacia Biotech) was subsequently added to cleave the MS protein from its GST fusion partner at 4°C for approximately 16 h.

Protein concentrations of the various enzyme extracts were determined by the Protein Assay Reagent (Bio-Rad, Singapore) according to the manufacturer's instructions. The soluble and purified protein extracts were separated on a 10% sodium dodecyl sulfate polyacrylamide gel by electrophoresis (SDS-PAGE) [13]. The relative amounts of MS protein expressed were determined by scanning densitometry using the Bio-Rad GS-700 Imaging Densitometer.

Enzymatic analysis

The MS assay was based on Ornston and Ornston [18]. The reaction was initiated by the addition of purified enzyme into the assay mixture containing 10 μM $MgCl_2$, 0.2 μM acetyl-CoA,

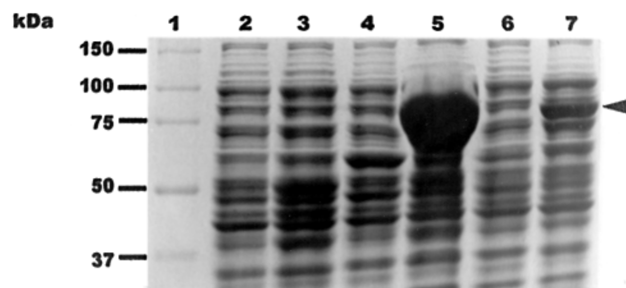

Figure 1 SDS-PAGE analysis of the soluble protein fractions of streptomycete MS obtained from *E. coli* BL21(DE3) after expression at 20°C. Lane 1, molecular weight markers; lane 2, *E. coli* BL21(DE3); lane 3, *E. coli* BL21(DE3)/pGEX-6P-1 (nonrecombinant); lane 4, nonfusion *S. coelicolor* MS; lane 5, pGEX-ScoeMS (fusion GST–*S. coelicolor* MS); lane 6, nonfusion *S. clavuligerus* MS; lane 7, pGEX-SclavMS (fusion GST–*S. clavuligerus* MS). The arrowhead indicates the position of the fusion GST–MS protein.

10 μM glyoxylate and 100 μM Tris–HCl buffer (pH 8), in a total volume of 0.9 ml, and stopped by 2 ml of 6 M urea. Color development was facilitated by the addition of 0.1 ml of 10 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) (DNB), followed by absorbance determination at 412 nm after 10 min. An increment of 4.53 absorbance units at this wavelength corresponded to the release of 1 μM CoA per 3 ml of reaction mixture.

Variable concentrations of $MgCl_2$ (0–20 μM), acetyl-CoA (0.01–0.08 μM) and glyoxylate (0.25–20 μM) were used in the determination of optimized reagent concentrations. The temperature and pH range for efficient enzyme activity were also probed by performing the MS assay at different temperatures (4–65°C) and pH (6.8–10°C). The kinetic parameters for each MS enzyme were determined for the substrates, acetyl-CoA and glyoxylate by Lineweaver–Burke analysis. The initial velocities were obtained at five different concentrations of acetyl-CoA, ranging from 0.01 to 0.1 μM in the presence of excess cosubstrate. The same experiment was carried out for glyoxylate, using concentrations ranging from 0.25 to 5 μM. The assay was performed in Tris–HCl buffer (pH 8) at room temperature and the amount of enzyme used was 0.6 μg. Data points were then used to plot Lineweaver–Burke graphs to

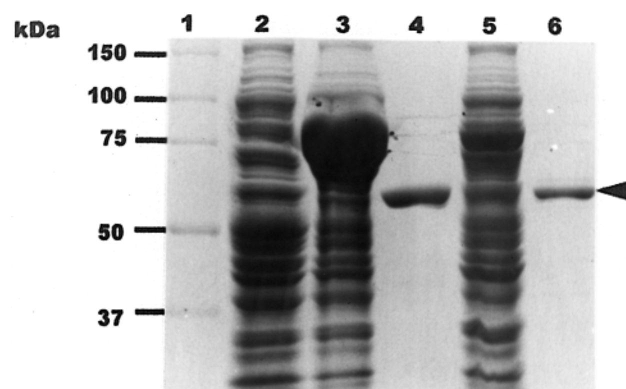

Figure 2 SDS-PAGE analysis of the purified protein fractions of streptomycete MS. Lane 1, molecular weight markers; lane 2, *E. coli* BL21(DE3)/pGEX-6P1 (nonrecombinant); lane 3, pGEX-ScoeMS; lane 4, purified *S. coelicolor* MS; lane 5, pGEX-SclavMS; lane 6, purified *S. clavuligerus* MS. The arrowhead indicates the position of the purified MS protein.

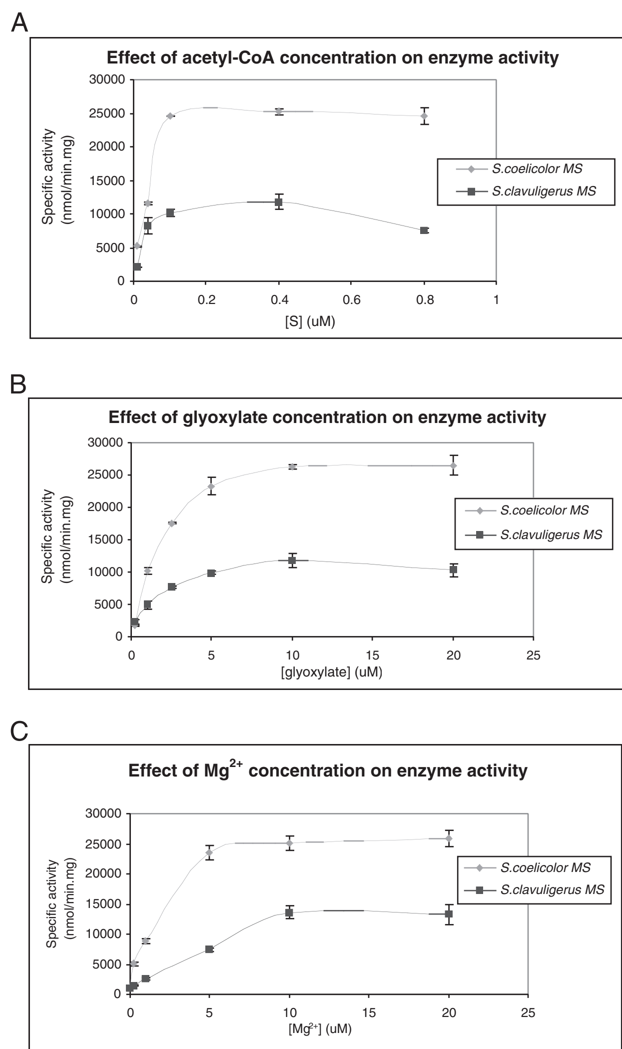

Figure 3 Effects of acetyl-CoA (A), glyoxylate (B) and $MgCl_2$ (C) concentrations on MS activity.

obtain the K_m values. Each experiment was performed in triplicate and repeated with the average values taken.

Sequence alignment and secondary structure prediction

Amino acid sequence alignment of MS was performed using the CLUSTAL W multiple sequence alignment program (version 1.7) [21]. Protein secondary structure predictions were performed using the self-optimized prediction method (SOPM) for protein secondary structure prediction program [5].

Results

Cloning and DNA sequencing

The *aceB* genes from *S. coelicolor* and *S. clavuligerus* were cloned into pCR®-BluntII-TOPO vectors and fully sequenced on both strands. Subsequently, the genes were subcloned into pGEX-6P-1, resulting in the recombinant constructs, pGEX-ScoeMS and pGEX-SclavMS, for *S. coelicolor* and *S. clavuligerus* MS, respectively.

Heterologous expression and purification

Expression studies of the recombinant constructs pGEX-ScoeMS and pGEX-SclavMS (Figure 1, Lanes 5 and 7) compared to the nonfusion MS constructs (Figure 1, Lanes 4 and 6) revealed that the soluble expression improved 9.5- and 10-fold for *S. coelicolor* and *S. clavuligerus* MS enzymes, respectively, as determined by scanning densitometry. The relative amount of soluble expression of pGEX-SclavMS was lower than that of pGEX-ScoeMS. Nevertheless, the use of the GST fusion vector allowed the expression of sufficient quantities of *S. clavuligerus* MS for enzymatic analysis. Purified MS enzymes, which were cleaved by PreScission™ Protease from the GST fusion partner, were obtained by affinity purification with Glutathione Sepharose 4B (Figure 2).

Enzymatic and kinetic analyses

Experiments were performed to determine the enzymatic characteristics of the purified MS enzymes. First, the optimum concentrations of acetyl-CoA and glyoxylate for both streptomycete MS enzymes were examined and both enzymes were shown to be maximally active at 0.1 μM acetyl-CoA and 10 μM glyoxylate (Figure 3A and B). We also observed that at high acetyl-CoA concentrations (eightfold higher than the optimal), a reduction of streptomycete MS activity had occurred, with the reduction being more pronounced in *S. clavuligerus* MS. Similarly for glyoxylate, a twofold increase in the optimal concentration also decreased the maximal specific activity of *S. clavuligerus* MS. The slight reduction of both MS activities at high acetyl-CoA and glyoxylate concentrations could be due to substrate inhibition [15]. Enzymatic inhibition by Li^+ and Na^+ was also reported previously [16]. These two cations are provided with the substrates, acetyl-CoA and glyoxylate, respectively, and the increased ionic strength might account for the reduction of MS activity at high substrate concentrations. Based on these results, we recommend the use of an excess concentration of 0.2 μM acetyl-CoA for the analysis of streptomycete MS activity, instead of 0.4 μM as suggested by Ornston and Ornston [18].

Studies on the effect of $MgCl_2$ concentration on MS activity revealed that the optimum $MgCl_2$ concentration was 5 and 10 μM for *S. coelicolor* and *S. clavuligerus* MS, respectively (Figure 3C).

Table 2 Enzymatic parameters of *S. coelicolor* and *S. clavuligerus* MS enzymes

MS	Optimum			Specific activity (nmol/mg min)	K_m (μM) Acetyl-CoA	K_m (μM) Glyoxylate
	$MgCl_2$ (μM)	Acetyl-CoA (μM)	Glyoxylate (μM)			
S. coelicolor	5	0.1	10	26,199	0.035	3.49
S. clavuligerus	10	0.1	10	11,821	0.029	0.59

K_m for acetyl-CoA and glyoxylate were determined for purified *S. coelicolor* and *S. clavuligerus* MS by extrapolation from the Lineweaver–Burke plot in the presence of saturating concentrations of cosubstrate and Mg^{2+}. Values indicated are means obtained from triplet measurements. The assay reactions were performed in Tris–HCl buffer (pH 8) at room temperature.

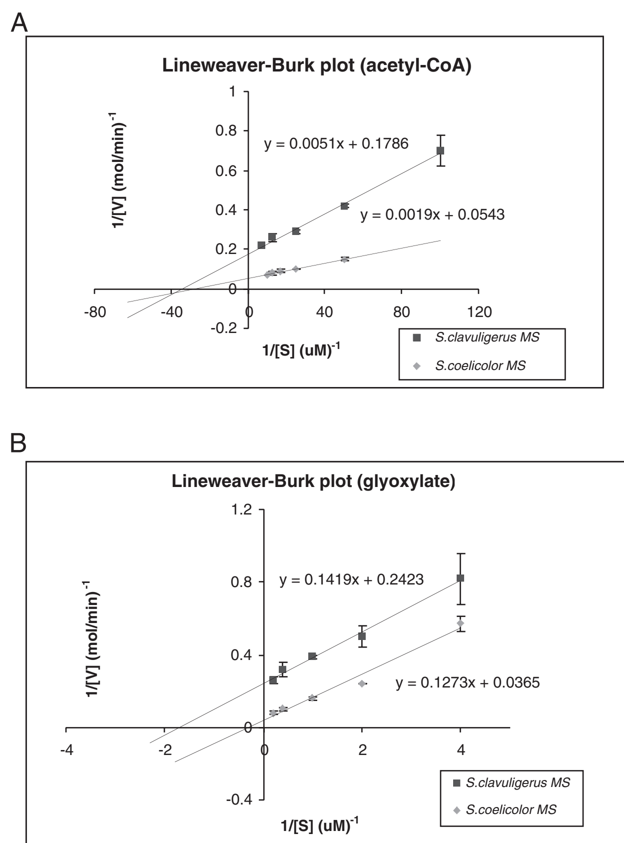

Figure 4 Double reciprocal plots for acetyl-CoA (A) and glyoxylate (B) of *S. coelicolor* and *S. clavuligerus* MS. The K_m values are shown in Table 2.

Mg^{2+} was absolutely required as a cofactor for the enzymatic reaction as there was a marked reduction of more than 90% in the specific activity of *S. coelicolor* and *S. clavuligerus* MS in the absence of added Mg^{2+}. Both streptomycete MS also showed a typical feature of mesophilic enzymes, which are able to function over a temperature range of 20–45°C. Similar to *S. arenae* MS, both MS enzymes in our study operated more efficiently in the pH range of 8–10, with significant reduction of specific activity at pH 7 and below [9] (data not shown). Using the optimized substrate and $MgCl_2$ concentrations, the average purified specific activities of *S. coelicolor* and *S. clavuligerus* MS at room temperature and pH 8 were determined to be 26,199 and 11,821 nmol/mg min, respectively (Table 2).

From linear Lineweaver–Burke double reciprocal plots, the K_m values for acetyl-CoA and glyoxylate displayed by *S. coelicolor* MS were 0.035 and 3.49 μM, respectively, while those displayed by *S. clavuligerus* MS were 0.029 and 0.59 μM, respectively (Figure 4A and B).

Discussion

As the use of fusion proteins was originally proposed for the ease of protein purification and immobilization, it was discovered that certain fusion partners could improve the solubility of the recombinant protein of interest [1]. In a comparative study of three different fusion partners, namely the maltose-binding protein (MBP), thioredoxin and GST, it was proposed that MBP was a far more effective solubilizing partner than the other two proteins [10]. However, in other reported studies, GST was also successfully used as a solubilizing partner, as in the case of streptavidin from *S. avidinii* and the Duffy binding protein from *Plasmodium vivax* [4,6]. In this study, we have also found that GST was effective in improving the soluble expression of both streptomycete MS enzymes. This could be due to GST being able to efficiently and rapidly reach a native conformation earlier than the MS enzymes, thus promoting and favoring the acquisition of the correct protein structure of its partner [1].

The two streptomycete MS enzymes share a high amino acid identity of 80% and a high homology of 83.7% in their secondary structure conformation. Although these two MS enzymes have close sequence and structural similarities, their enzymatic characteristics are not necessary identical, as shown in our study. For example, the average purified specific activity of *S. coelicolor* MS was consistently almost 2.5-fold higher when compared to *S. clavuligerus* MS. Our experiments also showed that the *S. clavuligerus* MS exhibited a K_m for glyoxylate that is sixfold lower than that of *S. coelicolor* MS, although there was no significant difference in the K_m values for acetyl-CoA. This indicates that *S. clavuligerus* MS may have a much higher binding affinity for glyoxylate. A further comparison of the K_m values for the two substrates displayed by these two streptomycete MS enzymes with those from another Gram-positive bacterium, *Corynebacterium glutamicum* [19], showed that the former were much lower. This suggests that the glyoxylate bypass may still operate effectively in *Streptomyces* spp. in a nutritionally poor environment where a carbon source is scarce.

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