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NOTE

Identification of Genes for Mycothiol Biosynthesis in *Streptomyces coelicolor* A3(2)

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Mycothiol is a low molecular weight thiol compound produced by a number of actinomycetes, and has been suggested to serve both anti-oxidative and detoxifying roles. To investigate the metabolism and the role of mycothiol in *Streptomyces coelicolor*, the biosynthetic genes (*mshA, B, C,* and *D*) were predicted based on sequence homology with the mycobacterial genes and confirmed experimentally. Disruption of the *mshA, C,* and *D* genes by PCR targeting mutagenesis resulted in no synthesis of mycothiol, whereas the *mshB* mutation reduced its level to about 10% of the wild type. The results indicate that the *mshA, C,* and *D* genes encode non-redundant biosynthetic enzymes, whereas the enzymatic activity of MshB (acetylase) is shared by at least one other gene product, most likely the *mca* gene product (amidase).

Keywords: mycothiol, *mshABCD, mca,* detoxification, *Streptomyces coelicolor* A3(2)

Many organisms, including most eukaryotes and gram-negative bacteria, possess glutathione as their major low molecular weight thiols for protection against oxidative stress and detoxification of xenobiotics. However, nearly all actinomycetes, especially mycobacteria and streptomycetes, possess mycothiol, 1-D-*myo*-inosityl 2-(*N*-acetyl-L-cysteinyl)amido-2-deoxy-α-D-glucopyranoside (MSH), instead of glutathione (Newton *et al.*, 1996). Mycothiol was reported as U17 in *Streptomyces clavuligerus* (Newton *et al.*, 1993), and the common name, mycothiol, was given after the subsequent study in *Mycobacterium bovis* (Spies *et al.*, 1994).

Four enzymes participate in the pathway of mycothiol biosynthesis (Fig. 1). MshA, a glycosyltransferase, makes disaccharide moiety, 1D-*myo*-inosityl 2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc-Ins, Newton *et al.*, 2003), from which the acetyl group is removed by MshB, a deacetylase. This forms 1D-*myo*-inosityl 2-amino-2-deoxy-α-D-glucopyranoside (GlcN-Ins, Newton *et al.*, 2000a; Rawat *et al.*, 2003). MshC, a ligase, links cysteine to GlcN-Ins (Sareen *et al.*, 2002); MshD, a mycothiol synthase, completes MSH by transferring an acetyl group from acetyl CoA to 1D-*myo*-inosityl 2-(L-cysteinyl)amido-2-deoxy-α-D- glucopyranoside (Cys-GlcN-Ins, Koledin *et al.*, 2002)

Glutathione is involved in the detoxification of endobiotic and xenobiotic electrophiles (Penninckx and Elskens, 1993; Shin *et al.*, 2003; Kim *et al.*, 2004), which suggests that mycothiol might also play a sim-

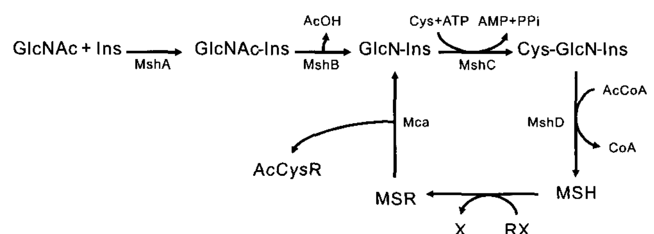

Fig. 1. The pathway for biosynthesis, recycling of mycothiol, and detoxification of xenobiotics.
Abbreviations used are: GlcNAc (2-amino-2-deoxy-α-D-glucopyranoside), Ins (1D-*myo*-inositol), GlcNAc-Ins (1D-*myo*-inosityl 2-acetamido-2-deoxy-α-D-glucopyranoside), GlcN-Ins (1D-*myo*- inosityl 2-amino-2-deoxy-α-D-glucopyranoside), Cys-GlcN-Ins [1D-*myo*-inosityl 2-(L-cysteinyl)amido-2-deoxy-α-D-glucopyranoside], MSH [mycothiol, 1D-*myo*-inosityl 2-(*N*-acetyl-L-cysteinyl)amido-2-deoxy-α-D-glucopyranoside], RX (electrophile), MSR (MSH conjugate of the electrophile), and AcCysR (mercapturic acid) (Newton *et al.*, 2002).

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ilar role. The first evidence for detoxification by MSH was MSH-dependent formaldehyde dehydrogenase (Misset- Smits *et al.*, 1997; Norin *et al.*, 1997), which was thought to be involved in the conversion of toxic formaldehyde to formic acid. Amidase (Mca) participates in a more general detoxification pathway against electrophiles. An electrophile (RX) is conjugated with MSH and forms MSR, which is cleaved by Mca to form AcCysR (mercapturic acid) and GlcN-Ins (Fig. 1). AcCysR is pumped out of the cytoplasm and GlcN-Ins is recycled for the biosynthesis of MSH (Rawat *et al.*, 2004). Mca was purified as an enzyme responsible for cleaving MSmB (MSH conjugated with an alkylating agent, monobromobimane) in *M. smegmatis* (Newton *et al.*, 2000b). It has broad substrate specificity which includes antibiotics. This suggests that this system might be important in antibiotic-producing bacteria. Mca is very similar to the deacetylase MshB, and the enzyme from *M. tuberculosis* was shown to have significant deacetylase activity (Steffek *et al.*, 2003). MSH reductase (Mtr), required for the maintenance of reduced MSH, was first identified as a glutathione reductase homologue (Cole *et al.*, 1998), and subsequent studies with purified Mtr from *M. smegmatis* (Patel *et al.*, 1998) and *M. tuberculosis* (Patel *et al.*, 1999) showed that it was very similar to glutathione reductase.

MSH-deficient mycobacterial mutants were shown to be sensitive to free radicals, alkylating agents, and a wide range of antibiotics, including erythromycin, azithromycin, vancomycin, penicillin G, rifamycin, and rifampin (Rawat *et al.*, 2002). The *mshB* mutant grew poorly on agar media that lacked catalase and oleic acid, and became sensitive to the toxic oxidant, cumene hydroperoxide, and to the antibiotic, rifampin (Buchmeier *et al.*, 2003). These findings suggest that MSH might play a crucial role in protection against various stresses.

Streptomycetes, which produce various antibiotics, raise interesting questions about the role of mycothiol because of the need to avoid the detrimental effect of antibiotics created in the cytoplasm. It was proposed that MSH-dependent detoxification protects against the antibiotics produced, their electrophilic precursors, or their oxidation products in antibiotic-producing organisms (Newton *et al.*, 2002). In this paper, we searched the homologues of the *mshA, B, C,* and *D* from the genome database of *S. coelicolor* and examined the disruption mutants of those genes.

Homologues of the genes involved in biosynthesis (*mshA, B, C,* and *D*) and detoxification (*mca*) were searched by BLAST in the *S. coelicolor* genome database at Sanger Center (www.sanger.ac.uk/cgi-bin/blast/submitblast/s_coelicolor), using the sequences of these genes in *Mycobacterium tuberculosis* (Rv0486, Rv1170, Rv2130c, Rv0819, and Rv1082) (Table 1). In each case, the results of the BLAST search contained multiple candidates. In particular, the homologues of *mshB* and *mca* overlapped with each other. In the case of *mshB*, SCO5126 had the highest score and SCO4967 had the second-highest score. In contrast, the closest homologue of *mca* was SCO4967 and the next-closest was SCO5126. In fact, *mshB* was identified using the sequence of *mca* (Newton *et al.*, 2000a), and it was reported that deacetylase and amidase act on the same type of chemical structure

Table 1. Blast search of *msh* and *mca* homologues

	Query	Homologues	Score	P(N)
mshA	Rv0486	SCO4204	784	1.3e-79
		SCO6185	420	4.9e-41
		SCO0962	225	7.7e-18
mshB	Rv1170	SCO5126	423	2.3e-41
		SCO4967	228	1.1e-20
		SCO7632	178	4.7e-14
mshC	Rv2130c	SCO1663	1181	1.1e-121
		SCO4235	541	7.3e-54
		SCO0780	76	0.22
mshD	Rv0819	SCO4151	481	1.7e-47
		SCO1545	109	2.6e-05
		SCO2379	82	0.0079
mca	Rv1082	SCO4967	693	5.7e-70
		SCO5126	370	9.7e-36
		SCO7632	295	8.6e-28

Each gene from *M. tuberculosis* was blasted against the genome sequence of *S. coelicolor*. The top three candidates of each BLAST result were presented.

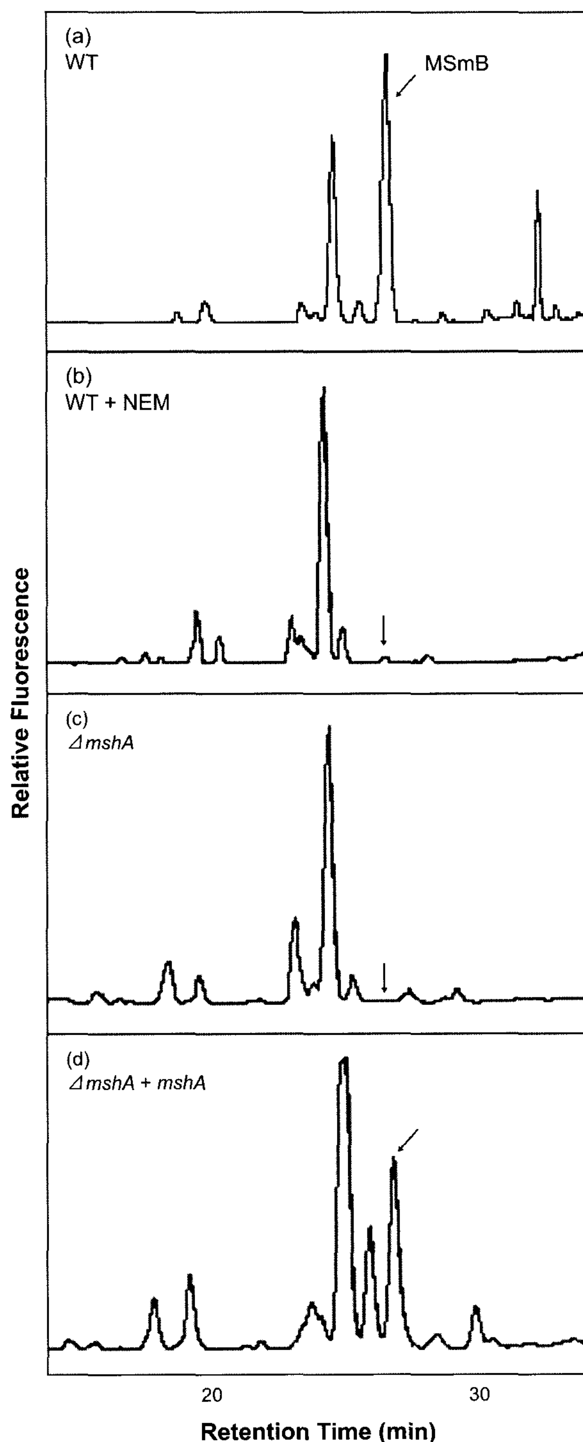

Fig. 2. HPLC analysis of mycothiol.
HPLC profiles of (a) wild-type, (b) wild-type plus NEM, (c) ΔSCO4204 (*mshA*), and (d) ΔSCO4204 complemented strains. When cells were labeled with monobromobimane (mBBr), MSH formed a complex with mBBr, to form MSmB (monobromobimane derivative of MSH). The MSmB peak was visible at 27 min in the wild-type cell extract, but was absent in the ΔSCO4204 mutant. It reappeared in the complemented strain. ΔSCO5126, ΔSCO1663, and ΔSCO4151 mutants also showed similar results. MSmB peaks are indicated by arrows.

(Steffek *et al.,* 2003). The candidates with the highest scores were selected for the construction of mutants; these were SCO4204 (*mshA*), SCO5126 (*mshB*), SCO1663 (*mshC*), and SCO4151 (*mshD*).

To test whether the selected candidates really participated in mycothiol biosynthesis, they were disrupted using the PCR targeting mutagenesis method (Gust *et al.*, 2003). In *E. coli* BW25113/pIJ790, each gene in the cosmid was replaced by an apramycin cassette that had homologous extensions flanking the target gene. This recombinant cosmid was transformed into *E. coli* ET12567/pUZ8002 (Flett *et al.*, 1997), from which it was conjugated into M145 cells. The recombination occurred between the cassette in the recombinant cosmid and the target gene of the chromosome. We selected candidates that were resistant to apramycin and sensitive to kanamycin because of the double-crossover and the absence of an integrated cosmid, and the presence of a cassette in place of the target gene was verified by PCR and Southern analysis. We found that all of the mutants grew as well as the wild-type in YEME liquid media. On R5 plates, deletion mutants of SCO4204 and SCO1663 differentiated more slowly than the wild-type (data not shown).

Mycothiol levels of these mutants grown in YEME to late exponential phase (OD 0.7~0.8) were determined by a modification of the method described by Fahey and his colleagues (Fahey and Newton, 1987; Newton *et al.*, 1996; Buchmeier *et al.*, 2003). Harvested cells were sonicated in 40 mM HEPES (pH 8.0) and mixed with an equal volume of acetonitrile containing 4 mM monobromobimane (Fluka). The samples were incubated at 60°C for 20 min in the dark, and they were then acidified with 5 μl of 5 N methanesulfonic acid. The cell debris was pelleted by centrifugation, and the supernatants were diluted before injection into a C18 HPLC column (Waters Atlantis™ dC18). MSH was eluted with gradients of buffer A (0.1% trifluoracetic acid in distilled water) and buffer B (0.1% TFA in methanol). The proportion of buffer B in continuous gradients was as follows; 10% at 0~5 min, 18% at 10 min, 37% at 35 min, 70% at 40 min, 100% at 42~43 min, 10% at 45~50 min, and reinjection. Control samples were treated with 5 mM N-ethylmaleimide (final concentration) at 60°C for 5 min. NEM was conjugated with the MSH thiol group, which could not be labeled with monobromobimane.

The mycothiol peak disappeared in the HPLC chromatogram of the SCO4204 (*mshA*) deletion mutant (Fig. 2C). Likewise, both the deletion of SCO1663 (*mshC*) and the deletion of SCO4151 (*mshD*) caused no production of MSH. However, in the SCO5126 deletion mutant, which lacked the homologue of *mshB*,

Table 2. Mycothiol levels in various mutants and their complemented strains with *msh* genes provided on pSET152 plasmids[a]

S. coelicolor strains	No plasmid[a]	+pSET152-*msh*[b]	+pSET152[c]
Wild-type	5.79±0.19	-	-
SCO4204 (*mshA*)	0	0.59±0.01	0
SCO5126 (*mshB*)	0.62±0.04	1.26±0.09	0.58±0.18
SCO1663 (*mshC*)	0	1.46±0.02	0
SCO4151 (*mshD*)	0	1.31±0.18	0

[a] The mycothiol level at late exponential phase was quantified (μmol/g [residual dry weight]).
[b] The genes that were disrupted in mutants were cloned into pSET152 vectors, which were then integrated into the chromosome for complementation.
[c] The parental pSET152 vector was integrated as a control.

mycothiol at late exponential phase remained at a level about ten-fold lower than in the wild-type (Table 2). It was reported that mycothiol production decreased in the *mshB* deletion mutant of *Mycobacterium smegmatis* (Rawat *et al.*, 2003), and the presence of residual mycothiol might be due to the deacetylase activity of amidase (Mca) (Steffek *et al.*, 2003). Deacetylase and amidase share a high level of similarity (36% identity in *M. tuberculosis*, 33.6% in *S. coelicolor*), which means they could compensate for each other to some extent. SCO4967 was also the candidate for the deacetylase, but the MSH level of the SCO4967 deletion mutant was nearly identical to that of the wild-type.

Complementation experiments were performed to confirm that the decrease in mycothiol production was directly caused by the disruption of these genes. Each gene was cloned into pSET152 vector, which contained a hygromycin cassette. The genes were then introduced into the corresponding disruptants by conjugation through *E. coli* ET12567/pUZ8002. Each cloned gene contained the entire ORF plus the 5' flanking region containing putative promoter; 123 nt upstream from the start codon for SCO4204 (*mshA*), 246 nt for SCO5126 (*mshB*), 184 nt for SCO1663 (*mshC*), and 159 nt for SCO4151 (*mshD*). Selection of candidates was performed by overlaying hygromycin on plates, and the integration of cloned pSET152 vector into chromosome was verified by PCR. Complemented strains were cultured until late exponential phase and were then harvested. When analyzed by HPLC, the mycothiol peak reappeared in the HPLC chromatogram (Fig. 2D), and the mycothiol levels of the complemented strains were restored to significant levels comparable to their parent strains (Table 2). When complemented strains were cultured until stationary phase (OD 1.4~1.5), their MSH levels approached the level observed in the wild-type cells at late exponential phase.

From this study, we showed that the mycothiol biosynthetic genes in *S. coelicolor* resemble their counterparts in mycobacteria. The *mshA* (SCO4204), *mshC* (SCO1663), and *mshD* (SCO4151) genes encode non-redundant biosynthetic enzymes, whereas *mshB* (SCO5126) encodes a deacetylase, the function of which could be partially provided by other enzymes, most likely Mca, mycothiol-S-conjugate amidase. Although the *msh* genes participated in the same biosynthetic pathway, they did not constitute an operon and were scattered in the *S. coelicolor* genome. The regulatory mechanism of these separated genes and the physiological role of MSH in *S. coelicolor* should be investigated further.

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