Cloning, characterization and expression of a gene encoding dihydroxyacetone synthase in *Mycobacterium* sp. strain JC1 DSM 3803

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Dihydroxyacetone synthase (DHAS) is a key enzyme involved in the assimilation of methanol in *Mycobacterium* sp. strain JC1 DSM 3803. The structural gene encoding DHAS in *Mycobacterium* sp. strain JC1 was cloned using random-primed probes synthesized after PCR with synthetic primers based on the amino acid sequences conserved in two yeast DHASs and several transketolases. The cloned gene, *dasS*, had an ORF of 2193 nt, encoding a protein with a calculated molecular mass of 78 197 Da. The deduced amino acid sequence of *dasS* contained an internal sequence of *Mycobacterium* sp. strain JC1 DHAS and exhibited 29.2 and 27.3% identity with those of *Candida boidinii* and *Hansenula polymorpha* enzymes, respectively. *Escherichia coli* transformed with the cloned gene produced a novel protein with a molecular mass of ~78 kDa, which cross-reacted with anti-DHAS antiserum and exhibited DHAS activity. Primer-extension analysis revealed that the transcriptional start site of the gene was the nucleotide A located 31 bp upstream from the *dasS* start codon. RT-PCR showed that *dasS* was transcribed as a monocistronic message. Northern hybridization and β -galactosidase assay with the putative promoter region of *dasS* revealed that the gene was transcribed only in cells growing on methanol. The expression of *dasS* in *Mycobacterium* sp. strain JC1 was free from catabolite repression.

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INTRODUCTION

It is generally known that several methylotrophic bacteria contain key enzymes of more than one methanol assimilation pathway, though not the yeast xylulose monophosphate (XuMP) pathway, and no methylotrophic yeast possesses the key enzymes for prokaryotic C₁ assimilation pathways such as the ribulose monophosphate (RuMP) and serine pathways and the Calvin cycle (Anthony, 1982; de Koning & Harder, 1992; Dijkhuizen *et al.*, 1992; Fig. 1).

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Abbreviations: CBB, Coomassie Brilliant Blue; DHAS, dihydroxyacetone synthase; rGTG, representative GTG; rORF, representative ORF; RuMP pathway, ribulose monophosphate pathway; SD sequence, Shine–Dalgarno sequence; TKT, transketolase; XuMP pathway, xylulose monophosphate pathway.

The GenBank/EMBL/DDBJ accession number for the *dasS* sequence of *Mycobacterium* sp. strain JC1 is AY007261.

A figure showing the expression of *dasS* in *E. coli* is available as supplementary data with the online version of this paper.

We have previously reported that the facultatively chemolithotrophic bacterium *Mycobacterium* sp. strain JC1 (Song *et al.*, 2002) is able to grow aerobically not only on carbon monoxide but also on methanol as sole carbon and energy sources (Ro *et al.*, 1997b). Grown on methanol the bacterium exhibits neither hexulose-6-phosphate synthase nor hydroxypyruvate reductase activity, but contains ribulose biphosphate carboxylase and dihydroxyacetone synthase (DHAS) activities, indicating that the bacterium employs a novel method of C₁ assimilation (Park *et al.*, 2003; Ro *et al.*, 1997a, b); i.e. the Calvin cycle and the eukaryote-specific XuMP pathway, instead of the prokaryote-specific RuMP and serine pathways, function in *Mycobacterium* sp. strain JC1 growing on methanol (Fig. 1). The assimilation of C₁ metabolites in *Mycobacterium* sp. strain JC1 has been found to begin with the XuMP pathway, followed by both the XuMP pathway and the Calvin cycle (Ro *et al.*, 1997a).

DHAS (EC 2.2.1.3) is the key enzyme responsible for formaldehyde fixation in methylotrophic organisms adapting to the XuMP pathway during growth on methanol as a sole source of carbon and energy (Anthony, 1982; de Koning & Harder, 1992; Dijkhuizen *et al.*, 1992). The

Fig. 1. Catabolic and anabolic pathways in methanol-utilizing micro-organisms. *Mycobacterium* sp. strain JC1 growing on methanol was found to exhibit enzyme activities for both the Calvin cycle and the eukaryote-specific XuMP pathway, but not for those of the RuMP and serine pathways, which have been found in prokaryotes only (Ro *et al.*, 1997a).

enzyme has been regarded as a special transketolase (TKT) because it is able to catalyse the transfer of glycoaldehyde from the donor xylulose 5-phosphate to the acceptor formaldehyde, the first intermediate produced during methanol oxidation (Fig. 1), to form dihydroxyacetone and glyceraldehyde 3-phosphate (Kato *et al.*, 1979; O'Connor & Quayle, 1980; Waites & Quayle, 1980, 1981). DHAS also has properties different from those of the classical TKT in several aspects: it is unstable (Bystrykh *et al.*, 1981; Waites & Quayle, 1980), it is present only in cells grown on methanol (Kato *et al.*, 1979; O'Connor & Quayle, 1980; Waites & Quayle, 1980), and it is active toward a wide range of aldehydes (Kato *et al.*, 1982).

The DHAS from *Mycobacterium* sp. strain JC1 is composed of two identical subunits of molecular mass ~73 kDa, and is present only in cells grown on methanol, as in the case of methylotrophic yeasts, although it shares no immunological properties with the yeast DHAS (Ro *et al.*, 1997a). These results, together with the finding that DHAS proteins are also present in several other methanol-grown mycobacteria (Park *et al.*, 2003), suggest that a mycobacterial group, including *Mycobacterium* sp. strain JC1, is an evolutionary bridge for C₁-compound assimilation between the prokaryotic and eukaryotic methylotrophs.

In this study, we cloned and characterized the gene for DHAS (*dasS*) of *Mycobacterium* sp. strain JC1 to investigate the diversity and evolutionary relationships among the prokaryotic and eukaryotic DHASs, and also to elucidate the basic transcriptional characteristics of the *Mycobacterium* sp. strain JC1 *dasS* gene.

METHODS

Bacterial strains, phages, plasmids and cultivation conditions.

The bacterial strains, phages and plasmids used in this work are described in Table 1. *Mycobacterium* sp. strain JC1 DSM 3803 (Song *et al.*, 2002) was grown at 30 °C in standard mineral base (SMB) medium supplemented with 0.5% (v/v) methanol, 0.5% (w/v) pyruvate, 0.8% (w/v) nutrient broth, or a gas mixture of 30% CO/70% air, as described previously (Ro *et al.*, 1997b). To test the effect of other substrates on the expression of the DHAS gene, the bacterium was cultivated in SMB medium supplemented with 0.5% (v/v) methanol in the presence of 0.5% (w/v) pyruvate or 0.8% (w/v) nutrient broth. *Escherichia coli* strains were grown at

Table 1. Bacterial strains, phages and plasmids used in this study

Bacterial strain, phage, or plasmid	Description	Source or reference
Bacteria		
<i>Mycobacterium</i> strain JC1	Wild-type (DSM 3803)	Song <i>et al.</i> (2002)
<i>E. coli</i>		
DH5 α	<i>supE44 lac169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	BRL
XL-1 Blue MRA (P2)	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i> (P2 lysogen)	Stratagene
BL21(DE3) pLysS	F ⁻ <i>ompT hsdS_B</i> (<i>r_B⁻ m_B⁻</i>) <i>gal dcm</i> (DE3) pLysS (Cm ^r)	Novagen
Phages		
Lambda EMBL3	<i>Bam</i> HI-digested arms	Promega
Lambda clone 603	Lambda EMBL3 clone containing <i>dasS</i> gene of <i>Mycobacterium</i> strain JC1	This study
Plasmids		
pBluescript II KS+	2.96 kb cloning or sequencing vector, Amp ^r	Stratagene
pSKB7	pBluescript II KS+ containing a 3639 bp <i>Bam</i> HI fragment from clone 603	This study
pET-22b(+)	5493 bp T7 expression vector, Amp ^r	Novagen
pED18	pET-22b(+) containing a 2193 bp <i>Nde</i> I- <i>Xho</i> I fragment corresponding to the <i>dasS</i> gene	This study
pCV77	7469 bp vector containing a promoterless <i>lacZ</i> , Kan ^r	MediImmune
pNBV1	5.8 kb high-copy-number plasmid with pAL5000 replicon derived from p16R1, Hyg ^r	Nathan <i>et al.</i> (1995)
pDAS1	pNBV1 harbouring a 3555 bp <i>Not</i> I- <i>Nhe</i> I fragment containing a promoterless <i>lacZ</i> gene from pCV77	This study
pDAS2	pDAS1 harbouring a 271 bp <i>Xba</i> I fragment containing a putative <i>dasS</i> promoter	This study

37 °C in Luria–Bertani medium. Growth was measured with a spectrophotometer by determination of turbidity at 436 nm.

DNA manipulation. Chromosomal DNA was isolated from the cells of *Mycobacterium* sp. strain JC1 harvested at early stationary growth phase according to the method of Goldberg & Ohman (1984). Plasmid DNAs were isolated from *E. coli* by the alkaline lysis method described by Sambrook *et al.* (1989). Electrotransformation of *E. coli* was performed with a Gene Pulser apparatus (Bio-Rad). Competent cells were prepared as described by Dower *et al.* (1988). Phage lysate preparation and phage DNA isolation were carried out as described by the manufacturer (Promega, 1995).

PCR. Two synthetic primers, FWD and REV, were synthesized to amplify the region covering the *dasS* gene of *Mycobacterium* sp. strain JC1, based on the amino acid sequences conserved in several TKTs and DHASs. Primer FWD was a 32-fold degenerate 20-mer (5'-ccgaattcGCCAARTCSGGYCA YCCRG-3'), with an 8-mer extension that included an *EcoRI* site (underlined lower-case type), derived from a consensus amino acid sequence AKSGHPG that is present near the N termini of the DHASs of *Hansenula polymorpha* (34 YGGGHPG 40; Janowicz *et al.*, 1985) and *Candida boidinii* (32 YEGGHPG 38; GenBank accession no. AAC83349), and the TKTs of *Ralstonia eutropha* (28 AKSGHPG 34; Schäferjohann *et al.*, 1993), *Rhodobacter sphaeroides* (27 AKSGHPG 33; Chen *et al.*, 1991), *E. coli* (22 AKSGHPG 28; GenBank accession no. CAA48166) and *Saccharomyces cerevisiae* (26 ANSGHPG 32; Fletcher *et al.*, 1992). Primer REV was a 16-fold degenerate 21-mer (5'-aaggatccCTGW-CCSAGCGGKCCGGTSGT-3'), with an 8-mer extension that included a *BamHI* site (underlined lower-case type), based on the identical amino acid sequence TTGPLGQ, which is found in the N-terminal regions of DHASs in *H. polymorpha* (126–132; Janowicz *et al.*, 1985) and *C. boidinii* (124–130; GenBank accession no. AAC83349), and those of TKTs in *R. eutropha* (113–119; Schäferjohann *et al.*, 1993), *R. sphaeroides* (118–124; Chen *et al.*, 1991), *E. coli* (113–119; GenBank accession no. CAA48166) and *S. cerevisiae* (114–120; Fletcher *et al.*, 1992). The PCR mixture contained 200 µM of each dNTP, 20 pmol of each primer, 100 ng template DNA and 0.5 U *Taq* polymerase in 10 µl reaction buffer (50 mM Tris-HCl, pH 8.3, 250 µg BSA ml⁻¹, 1%, w/v, Ficoll, 1.5 mM MgCl₂). Amplification was carried out in a Fast Air Thermal Capillary Cycler (Daehan Medical Systems) as follows: after primary denaturation for 40 s at 94 °C, 30 cycles each of denaturation for 10 s at 94 °C, annealing for 10 s at 62 °C and elongation for 30 s at 72 °C were performed, and then post-elongation was carried out for 60 s at 72 °C. The PCR products digested with *EcoRI* and *BamHI* were eluted from the agarose gel after electrophoresis, purified with the QIAquick gel extraction kit (Qiagen), and cloned into pBluescript II KS+ for sequencing or used as templates to synthesize random-primed probes labelled with [α -³²P]dCTP for Southern blotting and plaque hybridization.

Southern hybridization. DNAs digested with restriction enzymes were subjected to electrophoresis and then transferred to Hybond-N⁺ membranes (Amersham) by capillary blotting. Hybridization and washing were carried out at 68 °C, as recommended by the manufacturer. Detection of positive bands or plaques was performed according to the methods described by Sambrook *et al.* (1989).

Cloning and DNA sequencing. The *Mycobacterium* sp. strain JC1 DNA partially digested with *Sau3AI* was ligated to lambda EMBL3 DNA, packaged by using Gigapack III Packaging Extracts (Stratagene), and transduced into *E. coli* XL1-Blue MRA (P2) cells following the manufacturer's instructions. Plaque replicas were screened with the labelled random-primed probes.

DNA fragments in subclones and nested deletion clones were sequenced by the dideoxy chain-termination method (Sanger *et al.*,

1977) using a T7 Sequenase DNA sequencing kit (version 2.0, Amersham). DNA and deduced amino acid sequences were analysed using DNASIS and PROSIS programs (version 7.00, Hitachi Software Engineering), respectively. The BLAST program was used to search the protein sequence database at the National Center for Biotechnology Information.

RNA isolation. A 50 ml culture of cells was harvested at the mid-exponential growth phase, washed once with 10 ml of 40 mM Tris-HCl buffer, pH 7.5, and resuspended in 1 ml TRIzol reagent (Invitrogen). Cells in the suspension were then disrupted by agitation four times at maximum speed for 45 s using a FastPrep 120 instrument (Qbiogene). RNAs in the cell lysate were isolated using TRIzol reagent according to the manufacturer's instructions, dried at room temperature, and dissolved in 100 µl nuclease-free water (Promega). The Qiagen RNeasy kit with a DNase I (Promega) digestion step was used to clean up the RNA preparation according to the manufacturer's protocol.

Northern blot analysis and primer extension. For Northern blot analysis, total RNAs were separated by formaldehyde agarose gel electrophoresis and visualized by ethidium bromide staining (Sambrook *et al.*, 1989). RNAs were then transferred to Hybond-N⁺ nylon membranes by vacuum blotter, cross-linked by UV irradiation as recommended by the manufacturer, and hybridized with random-primed probes synthesized by using a 1.28 kb *SmaI* fragment of pSKB7 as template.

A primer-extension experiment was performed with the avian myoblastosis virus reverse transcriptase primer extension system (Promega) and a 30-mer oligonucleotide primer, 5'-CGAATTGGGC-TCGGCAAGGATCGCATCTGC-3', which is complementary to nucleotide positions 50–79 bp downstream of the *dasS* start codon, according to the method of Sambrook *et al.* (1989).

RT-PCR. Four synthetic primers, DasSF, DasSR, TalF and TalR, were synthesized to elucidate the transcription unit of the *dasS* gene by RT-PCR. DasSF and TalF were a 22-mer (5'-GCTTCGACCCCGCTAAGTCCTT-3') and a 20-mer (5'-CCACCCCAACCGTACAACCTT-3'), respectively, which correspond to the nucleotide positions 956–977 bp and 8–27 bp downstream of the *dasS* and the putative *tal* gene start codons, respectively. DasSR (5'-GAAAGCTGTCCACGGGCTG-3') and TalR (5'-CGATCGGGGCGAACAGATCA-3') were 20-mers that are complementary to nucleotides 1564–1583 bp and 270–289 bp downstream of the *dasS* and the putative *tal* start codons, respectively. The reaction mixture (10 µl) for reverse transcription contained 5 µg RNA, 50 pmol DasSR or TalR, 1 µl each of 2.5 mM dNTP, 1 µl of 0.1 M DTT and 200 U SuperScript II reverse transcriptase (RT; Invitrogen) in First-Strand buffer (Invitrogen). After incubation of the pre-hybridization mix (RNA and primer) for 5 min at 65 °C, all other components were added. Reverse transcription was carried out for 30 min at 42 °C. As no-RT controls, reactions were set up with all ingredients except the RT. The resulting cDNA that covers a part of *dasS* only (cDNA-DasSR), and the cDNA that possibly contains the whole *dasS* gene together with part of the putative *tal* gene (cDNA-TalR) that is transcribed in the same direction immediately downstream of the *dasS* gene, were directly used for PCR. The PCR mixture contained 200 µM of each dNTP, 20 pmol each of appropriate primers, 1 µl cDNA-DasSR or cDNA-TalR and 0.5 U *Taq* polymerase in 50 µl reaction buffer (50 mM Tris-HCl, pH 8.3, 250 µg BSA ml⁻¹, 1%, w/v, Ficoll, 1.5 mM MgCl₂). Amplification was carried out in a Mastercycler gradient (Eppendorf) as follows: after primary denaturation for 7 min at 95 °C, 30 cycles each of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C and elongation for 1 min at 72 °C were performed, and then post-elongation was carried out for 5 min at 72 °C.

Overproduction of the DHAS gene in *E. coli*. Based on the deduced amino acid sequence of DHAS, oligonucleotide primers corresponding to the amino acid sequences of the N (5'-ggaattcatATGCGGCCGCCAGAGG-3') and C termini (5'-ccgctcgagTTA-GTTGCTGCCGC-3') with additional *NdeI* and *XhoI* sites (underlined) at the 5' ends of N and C termini, respectively, were synthesized to achieve successful cloning of PCR products into the expression vector. The PCR mixture contained 250 μ M of each deoxynucleoside triphosphate, 20 pmol of each primer, 100 ng pSKB7 and 2.5 U *Taq* polymerase in 100 μ l reaction buffer. Amplification was performed in a thermal cycler (model 480, Perkin-Elmer) as follows: after initial denaturation at 94 °C for 15 min, 30 cycles each of denaturation at 94 °C for 1 min, annealing at 65 °C for 2 min and elongation at 72 °C for 1 min were carried out, and then post-elongation was performed at 72 °C for 20 min. The amplified products were digested with *NdeI* and *XhoI* and cloned into pET-22b(+) (Novagen) to produce a subclone pED18. pED18 harbouring a complete 2193 bp *dasS* gene was subsequently introduced into *E. coli* BL21(DE3) pLysS and induced for DHAS by 1 mM IPTG.

Determination of the internal amino acid sequence of DHAS. DHAS was purified as described previously (Ro *et al.*, 1997a). The enzyme preparation containing 200 μ g DHAS was concentrated with a Centricon-10 (Bio-Rad) and left for 30 min at 37 °C. The concentrate was then treated with TCA to a final concentration of 10% (w/v), left for 30 min at room temperature, and sedimented at 13 000 g for 15 min. The resulting pellet was dissolved in 100 μ l 1 $\times$ SDS sample buffer (Laemmli, 1970) and treated with 2 μ l of 2 M NaOH to adjust the pH to 7.8. The polypeptide fragments were then subjected to denaturing PAGE at a constant 80 V and electroblotted onto a PVDF membrane (Schleicher & Schuell). After brief staining and destaining, a deeply stained peptide band was selected and analysed for amino acid sequence by the method of Edman & Begg (1967) with a protein sequencer (Milligen 600B).

Construction of reporter plasmid and transformation. To amplify the putative *dasS* gene promoter region covering a direct repeat (GTCAGAACCGTCAGAA) present at 59–44 bp upstream of the *dasS* transcription start site, two synthetic primers, with a 10-mer extension that included an *XbaI* site (underlined lower-case type), designated dSF (5'-gctctagagcTGATTGATGGTTCCGCTCATT-3') and dSR (5'-gctctagagcCCTCGGTTGTCCAGTTCAACA-3'), which corresponded to the nucleotides 128–108 bp upstream and 123–143 bp downstream of the *dasS* transcription start site, respectively, were synthesized. The PCR mixture contained 200 μ M of each dNTP, 20 pmol of each primer, 1 μ g template DNA and 1 U iMax *Taq* polymerase in 20 μ l reaction buffer (Intron). Amplification was carried out in a Mastercycler gradient (Eppendorf) as follows: after primary denaturation for 300 s at 95 °C, 30 cycles each of denaturation for 60 s at 95 °C, annealing for 90 s at 60 °C and elongation for 30 s at 72 °C were performed, and then post-elongation was carried out for 300 s at 72 °C. The 271 bp PCR products were eluted from the agarose gel after electrophoresis, purified with the QIAquick gel extraction kit, and cloned into the *XbaI* site of a promoterless vector pDAS1 to create a translational fusion to the *lacZ* gene, resulting in a reporter plasmid pDAS2. The stable mycobacterial promoter-probing vector pDAS1 was made by incorporation of the blunted *NotI*–*NheI* fragment of pCV77 (MediImmune) containing a promoterless *lacZ* gene into pNBV1 (Nathan *et al.*, 1995) digested with *PvuII*.

The vectors were introduced into *Mycobacterium* sp. strain JC1 by electroporation with a Gene Pulser apparatus (Bio-Rad) at 2.5 kV, 800 Ω and 25 μ F.

Enzyme assay. DHAS activity was assayed at 30 °C by a modification (Ro *et al.*, 1997a) of the method of Kato *et al.* (1982)

using xylulose 5-phosphate, α -glycerophosphate dehydrogenase, triosephosphate isomerase, and cell-free extracts or partially purified DHAS. Cell-free extracts and partially purified DHAS expressed in *E. coli* transformants were prepared according to the method of Ro *et al.* (1997a). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μ mol NADH min⁻¹. Protein content was determined by the method of Lowry *et al.* (1951), using BSA as a standard.

β -Galactosidase assays were performed as described elsewhere (Miller, 1972). Transformants carrying pDAS1 and pDAS2 were grown at 37 °C in 0.8% (w/v) nutrient broth and SMB medium supplemented with 0.5% (v/v) methanol or a gas mixture of 30% CO/70% air. Cells were harvested at the late-exponential growth phase, washed once with 0.05 M phosphate buffer, pH 7.0, and resuspended in the same buffer. Cells in the suspension were then disrupted by sonic treatment, centrifuged at 15 000 g for 30 min, and the resulting supernatant was used for the assay. Activity was expressed as Miller units per microgram total cell protein.

Electrophoresis. Denaturing PAGE was carried out following the method of Laemmli (1970), with several modifications described by Kim *et al.* (1989). Proteins were stained with Coomassie Brilliant Blue (CBB) R-250 by a modification (Kim & Hegeman, 1981) of the method of Weber & Osborn (1969).

Immunoblotting. Immunoblot analysis was performed with an anti-DHAS antiserum (Ro *et al.*, 1997a) following the ECL (enhanced chemiluminescence) Western blot protocols (Amersham) after transfer of proteins in the denaturing gel to a nitrocellulose membrane (Hybond-ECL, Amersham).

RESULTS AND DISCUSSION

Cloning of the DHAS gene

Since we were unable to identify the N-terminal amino acid sequence of DHAS, possibly due to blocking of the N terminus of the enzyme, two primers, FWD and REV, were synthesized based on the amino acid sequences conserved in several TKTs and DHASs, and were used to amplify the DHAS gene of *Mycobacterium* sp. strain JC1.

Using the two primers, a 0.3 kb DNA fragment was amplified by PCR with *Mycobacterium* sp. strain JC1 genomic DNA as a template (data not shown). The DNA fragment was sequenced after cloning into pBluescript II KS+ and found to be highly homologous to the sequences in part of the 5'-end regions of DHAS and TKT genes in several yeasts and bacteria (data not shown). Southern hybridization of *Mycobacterium* sp. strain JC1 genomic DNA with random-primed probes synthesized based on the PCR product revealed that the probes hybridized to one each of *Bam*HI (3.7 kb), *Eco*RI (>20 kb), *Pst*I (5.6 kb), *Sac*I (7.2 kb) and *Sal*I (4.3 kb) fragments (data not shown).

Two positive clones containing the 3.7 kb *Bam*HI fragment were obtained by plaque hybridization of the lambda library with the random-primed probes. Among the positive clones, lambda 603, which contains an 18 kb

insert DNA, was selected for restriction mapping. Based on the restriction map of the 18 kb insert (Fig. 2), a subclone pSKB7 was constructed in pBluescript KS+ for sequence analysis using the 3.7 kb *Bam*HI fragment, which hybridized with the random-primed probes.

Sequencing of the *Bam*HI fragment revealed the presence of several potential ORFs beginning at different ATG and GTG codons but ending with the same translation stop codon TAA, resulting in several proteins with different numbers of deduced amino acid residues due to the variation in the N-terminal sequences. An ORF starting with GTG (representative GTG; rGTG) and coding for 730 amino acid residues, which is closest to the number of residues in the *H. polymorpha* (710 aa; Janowicz *et al.*, 1985) and *C. boidinii* (706 aa; Sakai *et al.*, 1998) DHASs, was considered as a representative ORF (rORF). Among several other potential start codons for ORFs sharing the rORF reading frame, close to the rGTG were an ATG (12u-ATG) and two GTGs (38u-GTG and 40u-GTG) located 12, 38 and 40 amino acids upstream of the rGTG, respectively, and a GTG (52d-GTG) and an ATG (64d-ATG) residing 52 and 64 amino acids downstream of the rGTG, respectively. The deduced amino acid sequence of 64d-ATG ORF is devoid of a conserved critical amino acid H60, which is required for proton transfer during catalysis (Janowicz *et al.*, 1985; Sakai *et al.*, 1998).

A potential Shine–Dalgarno (SD) sequence (AGAAG) was present 6 bp upstream of the rGTG. Another potential SD sequence GGA was also identified 2 and 4 nt upstream of the potential start codons 38u-GTG and 52d-GTG, respectively. No SD sequence was found in the 10 bp upstream of the 12u-ATG, 40u-GTG and 64d-ATG potential start codons.

Based on the number of residues and the presence or absence of potential SD sequences and the H60 amino acid, the rORF starting with rGTG was selected as a strong candidate for the *dasS* gene in *Mycobacterium* sp. strain

JC1. The *dasS* candidate consisted of 2193 bp and encoded a protein with a calculated molecular mass of 78 197 Da and a pI of 5.42. A part of the amino acid sequence (533 AIPGLDVVGPGDANQVGIAW 552) deduced from the nucleotide sequence of the ORF matched completely with the N-terminal amino acid sequence of an internal peptide fragment of purified DHAS protein. This, together with the calculated molecular mass of a protein encoded by the cloned gene and the estimated molecular mass of a subunit of the purified enzyme (~73 kDa; Ro *et al.*, 1997a), suggests that the cloned gene is the structural gene for DHAS.

Further analysis of the downstream region also identified an incomplete putative reading frame. The frame consists of 275 amino acids with 63.5% identity to the amino acid sequence of the N-terminal region of a *Mycobacterium tuberculosis* transaldolase (GenBank accession no. AL123456), and starts with the ATG located 103 bp downstream of the *dasS* stop codon TAA.

Identification of the rORF as *dasS*

Western blot analysis revealed that a protein with a molecular mass of ~78 kDa was produced in *E. coli* harbouring pED18 induced by IPTG and cross-reacted with antiserum raised against purified DHAS (Supplementary Fig. S1), indicating that the cloned 2193 bp *dasS* candidate is the DHAS structural gene *dasS*.

Crude cell-free extracts prepared from the IPTG-induced cells of *E. coli* harbouring pED18 showed no measurable DHAS activity [$<0.001 \mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$]. Enzyme activity was, however, detected from partially purified enzyme preparations such as the eluates obtained after phenyl-Sepharose [$0.013 \mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$] and DEAE-Sephacel [$0.02 \mu\text{mol NADH oxidized min}^{-1} (\text{mg protein})^{-1}$] ion-exchange chromatography, further supporting the assumption that the cloned *dasS* candidate is *dasS*. Cell-free

Fig. 2. Restriction map of an 18 kb insert DNA in lambda clone 603 containing the *Mycobacterium* sp. strain JC1 DHAS gene. *Bam*HI (B), *Not*I (N), *Pst*I (P), *Sac*I (C), *Sma*I (M) and *Sal*I (S) were used for restriction mapping. A detailed restriction map of the 3.7 kb *Bam*HI fragment in pBSK7 is shown under the 18 kb DNA map. The probe-binding site and a 1.28 kb *Sma*I fragment used as a probe for Northern blot hybridization are indicated under the 3.7 kb DNA fragment map. A complete ORF for DHAS (*dasS*) and an incomplete ORF possibly for a transaldolase (*tal*) are depicted as arrows with the direction of the arrowheads indicating the direction of transcription.

extracts prepared from *Mycobacterium* sp. strain JC1 grown on methanol used as a control oxidized $0.02 \mu\text{mol NADH min}^{-1} (\text{mg protein})^{-1}$.

Characteristics of the *dasS* candidate

The average G+C ratio of the *dasS* candidate was 67%. Codon usage analysis showed a strong codon bias for Gs and Cs in the third position; i.e. 56 out of 61 codons used were Gs and Cs. The amino acid sequence deduced from the nucleotide sequence of the *dasS* candidate was 29.2 and 27.3% identical to those of *C. boidinii* (Sakai *et al.*, 1998) and *H. polymorpha* (Janowicz *et al.*, 1985), respectively, which is in good agreement with an observation that *Mycobacterium* sp. strain JC1 DHAS shares no antigenic epitopes with the *C. boidinii* enzyme (Ro *et al.*, 1997a). The amino acid sequences deduced from the nucleotide sequences of the 12u-ATP and 52d-GTG ORFs were 28.7 and 27.4% and 29.6 and 27.9% identical to those of *C. boidinii* (Sakai *et al.*, 1998) and *H. polymorpha* (Janowicz *et al.*, 1985), respectively. The identity in the amino acid sequences of DHAS between *H. polymorpha* and *C. boidinii* is 69.1%, which is much higher than those between *Mycobacterium* sp. strain JC1 and the two yeasts. These results confirm the previous observations that the *Mycobacterium* sp. strain JC1 DHAS has antigenic groups in common with the DHASs of other mycobacteria but not with those of yeast (Park *et al.*, 2003; Ro *et al.*, 1997a), further supporting the previous notion that the gene for DHAS evolved independently in different evolutionary lines or at a very early time before divergence occurred, or that genetic exchange by wide-ranging mechanisms dispersed the common ancestral *dasS* gene to many different groups of methanol-utilizing organisms at some remote time. The results also suggest that the ability to assimilate methanol through the XuMP pathway in mycobacteria may have been acquired primarily by conservative independent evolution of an ancestral mycobacterium.

Analysis of aligned sequences of the deduced amino acid sequence of the *Mycobacterium* sp. strain JC1 *dasS* candidate, two yeast DHASs (Janowicz *et al.*, 1985; Sakai *et al.*, 1998) and an *M. tuberculosis* TKT (GenBank accession no. AL123456) confirmed that the protein encoded by the rORF is a type of TKT; it has been suggested before that DHAS is a special TKT that acts on xylulose 5-phosphate and formaldehyde (Kato *et al.*, 1979; O'Connor & Quayle, 1980; Ro *et al.*, 1997a; Waites & Quayle, 1980, 1981), and is expressed only in cells grown on methanol (Kato *et al.*, 1979; O'Connor & Quayle, 1980; Ro *et al.*, 1997a; Waites & Quayle, 1980). The primary structure of the *Mycobacterium* sp. strain JC1 DHAS candidate had residues relatively well conserved among the TKT family, such as those for thiamine pyrophosphate (TPP) binding [H100, G148, L150, G198, E202, D220, I226, H303, D420^s, L421^s, E460^s, F483^s, F486^s, Y489^s and H522^s; the superscript 's' indicates a residue from the second subunit (Hawkins *et al.*, 1989; Kato *et al.*, 1979; Robinson

& Chun, 1993; Wang *et al.*, 1997)]. Residues for divalent cation binding (D192, N222 and I224), for proton transfer during catalysis (H60, H134 and H522) and for subunit dimerization (P135, G516, E517, G519 and S700) were also conserved (Hawkins *et al.*, 1989; Lindqvist *et al.*, 1992; Sundström *et al.*, 1993; Robinson & Chun, 1993; Wang *et al.*, 1997). The residues for substrate binding (R125, E517 and D518) were identical or conservatively exchanged (Reizer *et al.*, 1993; Schäferjohann *et al.*, 1993).

Transcription of the *dasS* candidate

Primer extension placed the transcriptional start site of the *dasS* gene candidate at the A nucleotide located 31 bp upstream of the *dasS* start codon (Fig. 3). No additional bands outside the band corresponding to the A nucleotide were found on the gel. RT-PCR using the primer DasSR for cDNA-DasSR synthesis, and DasSR and DasSF for PCR with cDNA-DasSR as template, produced a 0.6 kb product (Fig. 4, lane 4) that is almost identical in size to the PCR product (628 bp) estimated from the nucleotide sequence. PCR using the primers TalR and TalF and the template cDNA-TalR obtained after reverse transcription with TalR also amplified a 0.3 kb fragment (Fig. 4, lane 6), which is close to the calculated size of the expected PCR product

Fig. 3. Identification of the transcriptional start site of the *dasS* gene. The transcriptional site of the *dasS* gene was identified by primer-extension mapping using a ^{32}P -labelled 30-mer oligonucleotide primer which was complementary to nucleotide positions 50–79 bp downstream of the *dasS* start codon, as described in Methods. Extension products were analysed (lane P) in parallel with the sequencing ladder (lanes G, A, T and C) primed with the same primer. The asterisk shows the transcriptional start site.

Fig. 4. RT-PCR for *dasS* gene transcription. (a) Primers used for RT-PCR. Primers DasSR and TalR were designed for reverse transcription to synthesize cDNAs covering only a part of the *dasS* gene (cDNA-DasSR), and both *dasS* and a part of the putative *tal* gene (cDNA-TalR), respectively, and also for PCR. DasSF and TalF were synthesized for PCR. (b) RT-PCR results. PCR was performed with DasSF/DasSR (lanes 1, 4 and 7), DasSF/TalR (lanes 2, 5 and 8) and TalF/TalR (lanes 3, 6 and 9) using genomic DNA (lanes 1, 2 and 3), cDNA-DasSR (lane 4), cDNA-TalR (lanes 5 and 6) and cDNA synthesized in the absence of reverse transcriptase (lanes 7, 8 and 9) as templates. M, molecular mass markers.

(282 bp). PCR with the primers TalR and DasSF and cDNA-TalR as template, however, produced no products (Fig. 4, lane 5). These results indicate that the *dasS* gene of *Mycobacterium* sp. strain JC1 is transcribed as a monocistronic message.

Putative promoter sequences for the -10 (TCATCG) and -35 regions (TATTAC), which are similar to those of the Mtb-T129 (TCAATA) and Msm-S5 (TAAGCT) genes, and to those of Mtb-T119 (TAGTAG) and Mtb-T150 (TATTGA), respectively (Bashyam *et al.*, 1996), were found upstream of the *dasS* transcriptional start site. A directly repeated sequence (underlined), GTCAGAAC-CGTCAGAA, which has been suggested to bind a transcriptional regulator (Ni & Westpheling, 1997), was also identified 59–44 bp upstream of the transcription start site. No repeated sequence was identified in the 150 bp upstream of the ORFs starting with 38u-GTG, 40u-GTG, 52d-GTG and 64d-ATG. These results, together with the number of amino acid residues and the presence or absence of SD sequence and H60, strongly support the supposition that the *dasS* candidate (rORF) starting with rGTG is the *dasS* gene in *Mycobacterium* sp. strain JC1.

The direct repeat located upstream of the *dasS* transcription start site is a potential site to which a regulator involved in the methanol-dependent expression of *dasS* gene binds (Ni & Westpheling, 1997). The observations that DHAS was present only in cells of *Mycobacterium* sp. strain JC1 growing on methanol (Ro *et al.*, 1997a, b) and that a highly similar directly repeated sequence (underlined), TCAGAACAT-CATCGTCAGAACAT, located 29–7 bp and 91–69 bp upstream of a potential -35 sequence and a translation start codon for *cbbL* in *Hydrogenophaga pseudoflava*, respectively, is protected from DNase when DNA fragments covering the direct repeat are treated with DNase I after incubation with the LysR-type transcriptional regulator

CbbR purified from *Hyd. pseudoflava* (S. N. Lee and Y. M. Kim, unpublished results) support this assumption.

Analysis of sequences downstream from the *dasS* candidate termination codon revealed a large stable GC-rich inverted-repeat sequence (underlined), CGCCGCGGGACGGCC-CGATGGCCGTCCC GCGGCG ($\Delta G^\circ = -47 \text{ kcal mol}^{-1}$; -197 kJ mol^{-1}), located 27–60 bp downstream of the stop codon and capable of forming a transcriptional termination structure. A poly-T segment was not found downstream of the inverted-repeat sequence. The stem-and-loop was followed by a long tract, at least 80 nt, free of double-stranded segments and containing several short GC-rich regions, suggesting that the termination of *dasS* candidate transcription may be rho-dependent.

Expression of the *dasS* gene in *Mycobacterium* sp. strain JC1

Since the DHAS of *Mycobacterium* sp. strain JC1 is expressed only in cells growing on methanol (Ro *et al.*, 1997a), efforts were made to determine at which level *dasS* expression was regulated. Northern hybridization was performed using total RNAs prepared from cells grown on CO, methanol and nutrient broth, and the random-primed probes synthesized by using a 1.28 kb *SmaI* fragment of pSKB7 as template. A band of ~2200 nt was detected in total RNA prepared from methanol-grown cells, but not the RNAs from CO- and nutrient broth-grown cells (Fig. 5), indicating that expression of *dasS* is regulated at the transcriptional level. This was further supported by a β -galactosidase assay with *Mycobacterium* sp. strain JC1 harbouring a reporter plasmid that contained the 271 bp PCR product covering the putative promoter region of the *dasS* gene including the 7 bp direct repeat and the putative -35 and -10 regions; i.e. the β -galactosidase activity in cells grown on methanol was 20- and 16-fold

Fig. 5. Northern hybridization of *Mycobacterium* sp. strain JC1 RNA. Northern hybridization was performed with random-primed probes synthesized by using a 1.28 kb *Sma*I fragment of pSKB7 as a template, as described in Methods. Total RNAs (20 µg each) were prepared from cells grown on CO (CO), methanol (ME) and nutrient broth (NB), and were visualized by ethidium bromide staining, as described in Methods.

higher than that of the cells grown on CO and nutrient broth, respectively (Fig. 6). The size of the RNA transcript detected in Northern hybridization with total RNAs from methanol-grown cells (Fig. 5) was slightly larger than that of the *dasS* gene (2193 bp), which supports monocistronic transcription of the *Mycobacterium* sp. strain JC1 *dasS* gene.

It is known that 0.5 % (w/v) pyruvate ($t_d=3.6$ h) and 0.8 % (w/v) nutrient broth ($t_d=3.6$ h) are more favourable than 0.5 % (v/v) methanol ($t_d=4.8$ h) for growth of *Mycobacterium* sp. strain JC1 (Ro *et al.*, 1997b). To

Fig. 6. β-Galactosidase assays of *dasS* promoter-*lacZ* fusions in *Mycobacterium* sp. strain JC1. β-Galactosidase activity was measured with cells harbouring pDAS1 (black bars) and pDAS2 (grey bars) that contain a promoterless *lacZ* gene and a putative *dasS* promoter-*lacZ* fusion fragment, respectively, after growth on CO (CO), methanol (ME) and nutrient broth (NB). β-Galactosidase activity (y axis) is expressed as nmol ONPG hydrolysed min⁻¹ (mg protein)⁻¹. The activity values represent the mean of five assays.

examine the effect of these compounds on the expression of *dasS*, cells were cultivated in SMB medium supplemented with 0.5 % (v/v) methanol in the presence of 0.5 % (w/v) pyruvate or 0.8 % (w/v) nutrient broth. Immunoblotting after denaturing PAGE of cell-free extracts prepared from cells grown on methanol in the presence of one of the two compounds showed that expression of *dasS* was not affected by favourable substrates, suggesting that the expression of *dasS* is free from catabolic repression.

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