Article No. jmbi.1999.2980 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (1999) 291, 531–536

JMB

COMMUNICATION

Identification of the *INO1* Gene of *Mycobacterium tuberculosis H37Rv* Reveals a Novel Class of Inositol-1-phosphate Synthase Enzyme

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1L-myo-inositol (inositol) is vital for the biogenesis of mycothiol, phosphatidylinositol and glycosylphosphatidylinositol anchors linked to complex carbohydrates in *Mycobacterium tuberculosis*. All these cellular components are thought to play important roles in host-pathogen interactions and in the survival of the pathogen within the host. However, the inositol biosynthetic pathway in *M. tuberculosis* is not known. To delineate the pathways for inositol formation, we employed a unique combination of tertiary structure prediction and yeast-based functional assays. Here, we describe the identification of the gene for mycobacterial INO1 that encodes inositol-1-phosphate synthase distinct in many respects from the eukaryotic analogues.

Keywords: INO1; inositol-1-phosphate synthase; inositol; *Mycobacterium tuberculosis*; sequence threading

Inositol is an essential metabolite in all eukaryotic cells, but is found rarely in prokaryotes. Those prokaryotic species in which inositol has been found to occur include *Streptomyces*, ice nucleating bacteria, and mycobacteria (Pittner *et al*., 1979; Kozlof *et al*., 1991; Bornemann *et al*., 1997; Parish *et al*., 1997). The mycobacterial cellular envelope contains a high proportion of phosphatidylinositol (PI), phosphatidylinositol mannoside (PIM), and glycosylphosphatidylinositol (GPI) anchor linked to arabinomannan (LAM) and mannan (LM) (Chatterjee *et al*., 1992; Hunter *et al*., 1990). These cellular components, LAM in particular, are thought to play crucial roles in the host-pathogen interaction and are particularly important for the survival of the pathogen in the hostile environment within the host (Sibley *et al*., 1988; Chan *et al*., 1991; Schlesinger *et al*., 1994). LAM has also been known to participate in the receptor-mediated phagocytosis of *Mycobacterium tuberculosis*, in addition to modulating the host immune response (Besra *et al*., 1997). The non-reducing termini of a variety of LAMs from rapidly growing strains of mycobacteria have been demonstrated to contain inositol phosphate capping (Khoo *et al*., 1995). Moreover in *Mycobacterium* sp., inositol is a precursor of another important cellular metabolite, mycothiol. Mycothiol is a thiol required for maintaining the intracellular reducing environment (Bornemann *et al*., 1997; Newton *et al*., 1996). Despite the important role played by inositol, the pathway for its biosynthesis has not yet been characterized in *M. tuberculosis* (Salman *et al*., 1999).

Studies with eukaryotic systems have revealed that the sole pathway for inositol biosynthesis involves two steps. In the first step, glucose-6-phosphate to inositol-1-phosphate conversion takes place through a complex reaction mediated by a single enzyme, inositol-1-phosphate synthase (EC 5.5.1.4). In the second step, inositol-1-phosphate is dephosphorylated by inositol-1-phosphatase (EC 3.1.3.25) (Chen & Charalompaus, 1964, 1965; Majumdar *et al*., 1997). The free inositol thus synthesized is subsequently used by the host as a precursor for generating inositol derived metabolities.

The first enzyme in the biosynthesis of inositol, inositol-1-phosphate synthase, has been shown to be essential for the growth of yeast and fungi. Disrupting the *INO1* gene in *Saccharomyces cerevisiae*, therefore, leads to cell death unless it is supplemented with exogenous inositol (Culbertson &

Abbreviations used: INO1p, inositol-1-phosphate synthase; ORF, open reading frame.

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0022-2836/99/330531–6 $30.00/0

Henry, 1975). As *Mycobacterium* is an inositol prototroph, it is likely to be capable of synthesizing inositol *de novo*. In this study we have sought to examine the presence of inositol-1-phosphate synthase in *M. tuberculosis*, and to determine if the pathway of inositol biosynthesis is common in eukaryotes and prokaryotes.

Publicly available *Mycobacterial* genome database was searched with the BLAST program (Altschul *et al*., 1997) using the *S. cerevisiae* inositol-1-phosphate synthase (INO1p) amino acid sequence. The search revealed a very low degree of sequence homology to the Rv0046c open reading frame (ORF) of the *M. tuberculosis H37Rv* genome coding for a 367 amino acid residue-long protein (Cole *et al*., 1998). The homology was sufficiently low to escape it being identified as the putative *Mycobacterial* inositol-1-phosphate synthase by standard computational methods. The percentage identity between *S. cerevisiae* INO1p and the protein product of Rv0046c was less than 15 %. Moreover, the sulfhydryl groups known to be important for the enzymatic activity of eukaryotic INO1p (Gumber *et al*., 1984) were not conserved in Rv0046c, giving rise to the speculation that Rv0046c may not possess the same activity. Thus, the assignment of the inositol-1-phosphate synthase function to the protein product of Rv0046c would not have been possible with the sequence data alone.

Inositol-1-phosphate synthase is known to utilize NAD as the cofactor (Majumdar *et al*., 1997). We therefore searched for a putative NAD binding sequence in the Rv0046c protein. However, neither the presence of the signature GXGXXG motif (Wierenga *et al*., 1986) nor an exhaustive search through the PROSITE database (Hofmann *et al*., 1999) revealed any nucleotide binding motifs in the Rv0046c sequence. On the other hand, a tertiary structure prediction by threading the sequence onto known protein folds (Fischer *et al*., 1996) revealed a Rossmann fold in the N-terminal half of the Rv0046c protein sequence. The mycobacterial INO1p exhibited striking similarity to diamino pimelate (DAP) dehydrogenase NADP binding domain (Scapin *et al*., 1996) with a Z-score of 7.99. This score is significantly higher than the confidence threshold limit set by the method, i.e. 4.8(±1). Interestingly, two arginine residues involved in 2′-phosphate binding in DAP dehydrogenase (Figure 1) are replaced by alanine residues in Rv0046c protein, thereby suggesting that Rv0046c may bind to NAD instead of NADP. Moreover, the sequence region predicted to be involved in nucleotide binding appeared to be highly conserved among the homologous prokaryotic and eukaryotic sequences (Figure 2). The threading therefore suggested that the N-terminal domain of Rv0046c might be functionally important for nucleotide binding.

In order to examine if the 367 amino acid residue-long ORF might be coding for an INO1 enzyme, primers were designed to amplify the Rv0046c coding gene by using a standard polymerase chain reaction (PCR) protocol. *M. tuberculosis H37Rv* chromosomal DNA was used as the template. The PCR product so obtained was verified by DNA sequencing. The PCR amplified gene product was subsequently cloned downstream of a strong, constitutively expressed yeast glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter of an yeast multicopy expression vector p426GPD (Mumberg *et al*., 1995; Sambrook *et al*., 1989) and the resulting plasmid was designated pBM002.

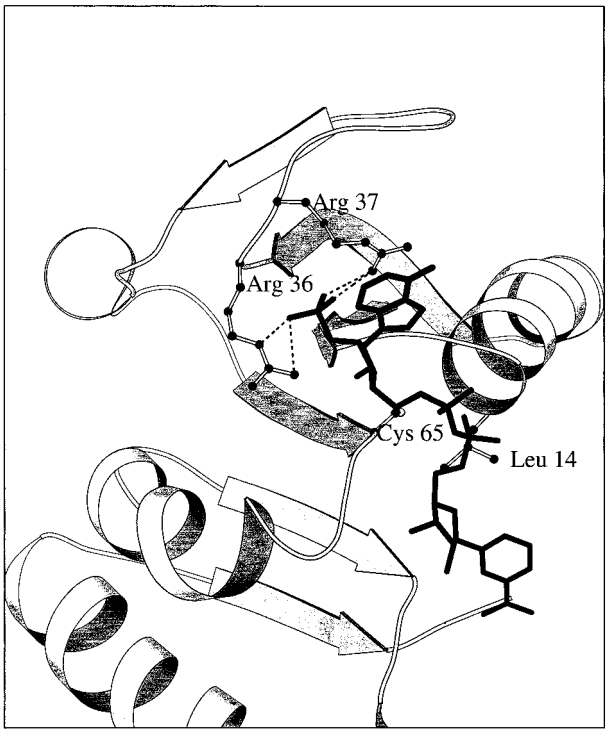
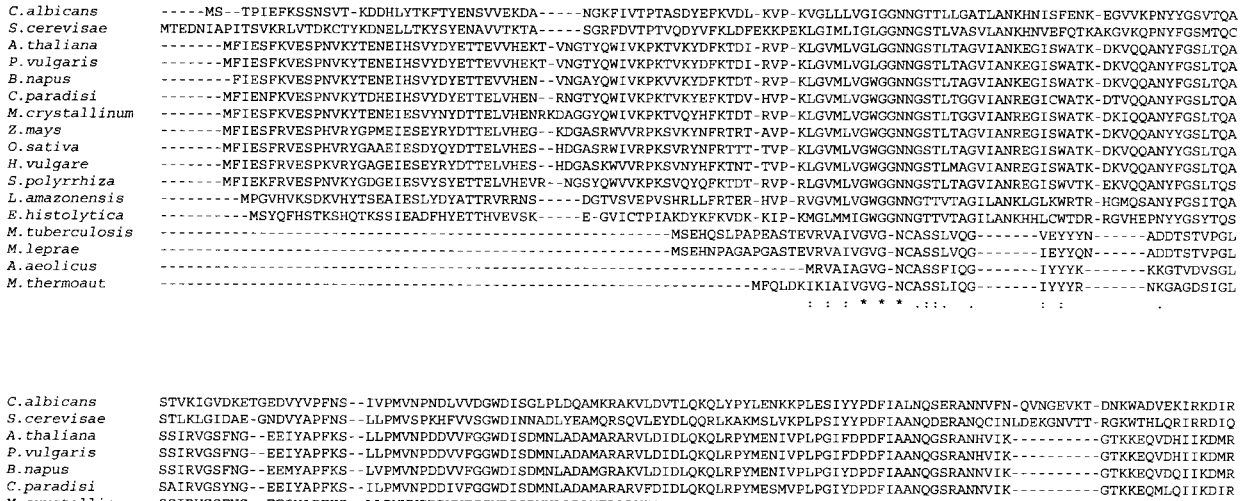
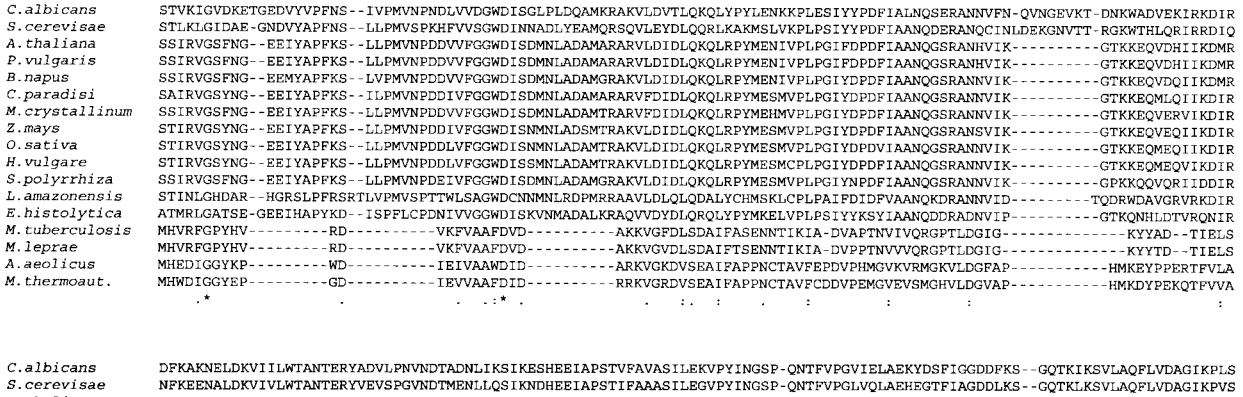
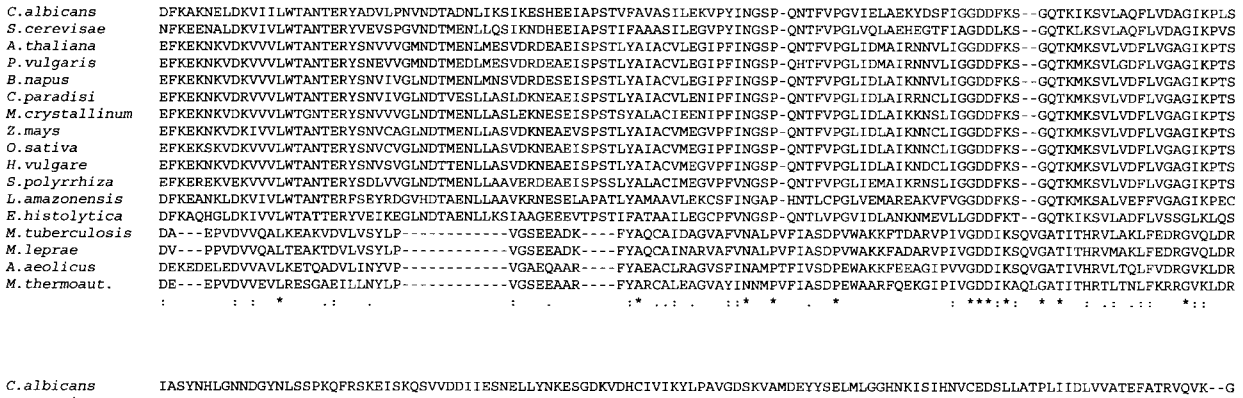


Figure 1. The binding mode of NADP as observed in diamino pimelate dehydrogenase drawn using MOLSCRIPT (Kraulis, 1991). The two arginine residues involved in binding the 2′-phosphate are replaced by alanine residues in *M. tuberculosis* INO1p sequence, thereby suggesting that the mycobacterial enzyme may bind NAD rather than NADP. The Leu residue shown is the conserved site of a Cys residue in prokaryotic INO1p sequences.

A yeast-based functional assay system was employed to determine if the ORF of Rv0046c indeed encoded an inositol-1-phosphate synthase enzyme. The assay was explored through functionally complementing the gene for yeast *INO1* deletion mutation in *S. cerevisiae*, FY250 (*MATα trp1 his3 ura3 leu2 Δinol::HIS3*). This yeast strain is auxotrophic for inositol due to the disrupted *INO1*. The strain FY250 was transformed with plasmid pBM002 harboring mycobacterial putative *INO1* gene and with the vector p426GPD as a control using standard protocols (Ito *et al*., 1983). Transformants bearing pBM002 construct allowed growth on medium lacking inositol (Figure 3), thereby suggesting that Rv0046c is capable of functionally complementing the yeast *ino1* mutation.







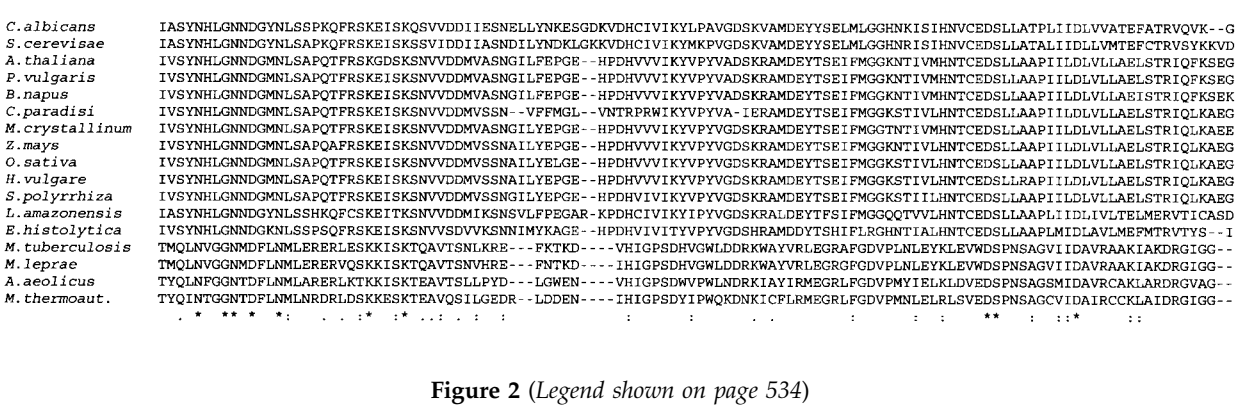


Figure 2 (*Legend shown on page 534*)

Further examination was carried out in liquid medium to ascertain the growth characteristics of the transformants harboring pBM002. The growth rate was examined in two separate defined media, one containing inositol but lacking uracil, and the other lacking both uracil and inositol. The strain FY250 containing the vector p426GPD was used as a control in both the media. As anticipated, the control transformants did not grow in the medium without inositol. However, they grew normally in a synthetic defined medium supplemented with 10 μM inositol with a doubling time of approximately 4.5 hours. The same host FY250 harboring the recombinant plasmid pBM002, on the

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C.albicans       PGKSD----YDELYPVASLLSYWLKAPLARPGFKPINGLNKQRQQLVNLLSVLVGLPIDNELRFERILK----------
S.cerevisae      PVKEDAG-KFENFYPVLTFLSYWLKAPLTRPGFHPVNGLNKQRTALENFLRLLIGLPSQNELRFEERLL----------
A.thaliana       EGK------FHSFHPVATILSYLTKAPLVPPGTPVINALSKQRAMLENIMRACVGLAPENNMIMEFK------------
P.vulgaris       EGK------FHSFHPVATILSYLTKAPLVPPGTPVINALSKQRAMLENIMRACVGLAPENNMIMEFK------------
B.napus          EGK------FHSFHPVATKLSYLTKAPLVPPGTPVVNALSKQRAMLENILRACVGLAPENNMILEYK------------
C.paradisi       EGK------FHSFHPVATILSYLTKAPLVPPGTPVVNALSKQRAMLENILRACVGLAPENNMILEYK------------
M.crystallinum   EDK------FHSFHPVATILSYLTKAPLVPPGTPVVNALSKQRAMLENILRACVGLAPENNMILEYK------------
Z.mays           EDK------FHSFHPVATILSYLTKAPLVPPGTPVVNALAKQRAMLENIMRACVGLAPENNMILEYK------------
O.sativa         EEK------FHSFHPVATILSYLTKAPLVPPGTPVVNALAKQRAMLENIMRACVGLAPENNMILEYK------------
H.vulgare        EDK------LHSFHPVATILSYLTKAPLVPPGTPVVNALAKQRAMLENIMRACVGLAPENNMILEYK------------
S.polyrrhiza     ESK------FHSFHPVASILSYLSKAPLVPPGTPVVNALSKQRAMLENILRACVGLAPENNMILEYK------------
L.amazonensis    ELKAPSPVSFEHMETVLSILSYLLKAPAVPEGTPVVNALNRQRAAIENLLRAMIGLPADSNMLLECRVPFMREEHVNGK
E.histolytica    DGKE-----FKNFNSVMSMISYLLKAPVVPKGSPVINALFKQRECLDNFFRALLGLPCDNHLLLEGKTEHFF-------
M.tuberculosis   ----------P----VIPASAYLMKSP------PEQLPDDIARAQLEEFIIG-------------------------
M.leprae         ----------P----VIPASSYLMKSP------PQQLPDEIARAQLEEFIIDTK-----------------------
A.aeolicus       ----------P----LYSISAYTMKHP------PIQYPDWQAKKMVEEFIRGERER---------------------
M.thermoaut.     ----------P----LTSISSYTMKHP------PVQYTDDVAARMVDEFIAGERER---------------------
                               : .  :*  * *            : :::
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Figure 2. Amino acids sequence alignment of *M. tuberculosis H37Rv* predicted *INO1* translational product (Rv0046c) and comparison of the same in single letter code with *INO1* ORFs of different organisms. Asterisks (*) indicate identity, dots (·) indicate similarity, while colons (:) indicate strong similarity. The sequence alignment shows two distinct clusters, one of which represents the eukaryotic enzymes.

other hand, exhibited normal growth pattern in the medium lacking exogenously added inositol. It had a doubling time of 4.5 hours, similar to the p426GPD containing transformant grown in inositol-containing medium. Thus, the growth characteristics strongly suggested that Rv0046c encodes inositol-1-phosphate synthase activity.

In order to confirm further that pBM002 harbors the functionally active mycobacterial inositol-1-phosphate synthase, inositol excretion was detected by following the procedure by Greenberg *et al*., 1982). To detect inositol excretion, randomly picked yeast transformants were spotted on agar plates lacking inositol. A lawn of the yeast indicator strain BRS1005 (*MATa/MATα*, *ade1/ade1*, *ino1-13/ino1-13*) was spread on the same plate prior to spotting the transformants. Inositol auxotrophic yeast strain FY250 containing pBM002 could be distinctly seen with red halo surrounding them. The halo is generated by the excreted inositol which permits the growth of the red indicator strain (BRS1005), which is also an inositol auxotroph (data not shown). Thus, FY250 transformed with pBM002 is clearly capable of synthesizing inositol and excreting it into the medium.

The functional identification of Rv0046c reveals a novel class of prokaryotic inositol-1-phosphate synthase. The 367 residue-long enzyme is considerably shorter than the eukaryotic analogues which are at least 500 to 550 residues long. The Rossmann fold domain successfully identified, not by conventional approaches but exclusively through sequence threading of known protein folds, indicated that it may be involved in nucleotide binding. The absence of conserved catalytic sulfhydryl groups, as has been shown to be critical in the plant and other eukaryotic enzymes, was the most significant factor for the inability of standard computational procedures to unambiguously establish the mycobacterial *INO1* function (Gumber *et al*., 1984; Majumdar *et al*., 1997). Although the critical sufhydryl group does not appear to be conserved in the Rv0046c sequence, the presence of a Cys residue, Cys26 (equivalent to Leu14 in the DAP

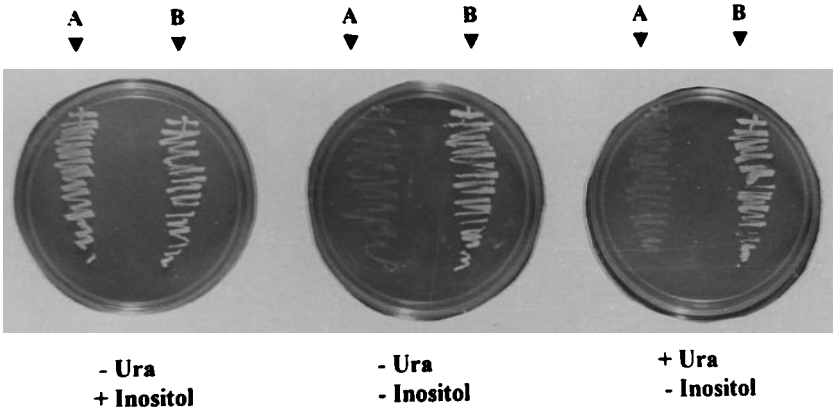

Figure 3. Growth pattern of the inositol-requiring *S. cerevisiae* strain FY250 transformed with (a) p426GPD as control and (b) *Mtb INO1* in pBM002. All the constructs were selected first in a synthetic medium containing inositol (+inositol) but lacking uracil (−ura), while the cloned gene was subsequently selected in the medium lacking inositol (−inositol). Thus, the transformants were grown on defined synthetic medium (Bachhawat *et al*., 1995) containing plus inositol minus uracil (+inositol, −ura), minus inositol minus uracil (−inositol, −ura), and minus inositol plus uracil (−inositol, +ura). The concentration of inositol was 10 μM.

structure as shown in Figure 1) next to the predicted nucleotide binding site suggests that Cys26 may take part in the proposed oxidation-reduction reaction. Interestingly, Cys26 appears to be conserved in all other prokaryotic homologous sequences (Figure 2). Also, the DAP dehydrogenase structure shows the presence of an important Cys65 residue very close to Leu14 (Scapin *et al*., 1996), reinforcing our prediction that Cys26 may act as the catalytic redox center. This prediction can be further tested by site-directed mutagenesis.

In conclusion, we have cloned the inositol-1-phosphate synthase from *M. tuberculosis H37Rv* and demonstrated that it can functionally compensate for the yeast *INO1* mutation. The *INO1* of *Mycobacterium* and other homologous prokaryotic genes revealed a distinct class of this enzyme. Although our demonstration that Rv0046c encodes an *INO1* activity, this does not rule out the possibility that another polypeptide in *M. tuberculosis* may play a similar role. Generating an *INO1* knock-out would conclusively demonstrate the same. Considering the important role of inositol in a variety of metabolic pathways in *M. tuberculosis* and the difference in the prokaryotic and eukaryotic enzymes, we suggest that the enzyme could be a potentially attractive drug target.

Acknowledgements

We thank Jaya Tyagi for the *M. tuberculosis H37Rv* chromosomal DNA. We are grateful to Susan A. Henry for providing us with the yeast strains FY250 and BRS 1005 and also to Martin Funk for providing us with the pGPD426 construct. We thank Anand Bachhawat for many useful discussions and suggestions for the improvement of the manuscript. The work was supported by IMTECH and CSIR.

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Edited by M. Yaniv

(Received 9 March 1999; received in revised form 24 June 1999; accepted 28 June 1999)