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NOTES

The *MTCY428.08* Gene of *Mycobacterium tuberculosis* Codes for NAD^+ Synthetase

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The product of the *MTCY428.08* gene of *Mycobacterium tuberculosis* shows sequence homology with several NAD^+ synthetases. The *MTCY428.08* gene was cloned into the expression vectors pGEX-4T-1 and pET-15b. Expression in *Escherichia coli* led to overproduction of glutathione *S*-transferase fused and His_6-tagged gene products, which were enzymatically assayed for NAD synthetase activity. Our results demonstrate that the *MTCY428.08* gene of *M. tuberculosis* is the structural gene for NAD^+ synthetase.

NAD^+ is a vital and ubiquitous coenzyme involved in a variety of biochemical processes, comprising not only redox reactions but also DNA repair, DNA recombination, and ADP protein ribosylation (7, 17). NAD^+ biosynthesis is therefore crucial in all living organisms and can be pursued through a de novo pathway or through a pyridine nucleotide salvage pathway (7, 18).

The ubiquitous enzyme NAD^+ synthetase (EC 6.3.5.1) catalyzes the last step in NAD^+ biosynthesis transforming deamido-NAD^+ into the final product NAD^+ by a two step reaction: (i) first it activates deamido-NAD^+ through the formation of an adenylated NAD^+ in the presence of ATP and magnesium, and then (ii) an ammonia molecule attacks the adenylated intermediate leading to NAD^+ (15).

NAD^+ synthetase belongs to the amidotransferase family, whose members are responsible for the transfer of the amide nitrogen of glutamine on different substrates in a large number of biochemical processes (20). Amidotransferases are characterized by the presence of two distinct domains, which may either belong to the same polypeptide chain or exist as independent subunits. A glutamine amide transfer (GAT) domain is responsible for the ability to use glutamine as nitrogen source, whereas a synthetase/synthase domain confers the specificity and catalyzes the transfer of ammonia to the substrate. Amidotransferases can be grouped into two families depending on their GAT domains: (i) G-type amidotransferases having a Cys-His-Glu catalytic triad and (ii) F-type or Ntn (N-terminal nucleophile) amidotransferases possessing a conserved N-terminal cysteine (20).

NAD^+ synthetase from *Bacillus subtilis* has been extensively characterized and reported to be strictly ammonia dependent (10), even if the existence of a yet unknown second subunit, bearing the GAT domain, cannot be excluded. The three-dimensional structure of the *B. subtilis* enzyme in its free form and in complex with ATP has also been reported (12), allowing the assessing of catalytically important residues and confirming the early proposal that NAD^+ synthetase, GMP synthetase, asparagine synthetase, and arginosuccinate synthetase constitute a new class of N-type ATP pyrophosphatases (ATP-PPases), characterized by the conserved fingerprint sequence S-G-G-X-D (16).

The major role played by NAD^+ synthetase in cellular metabolism is underlined by previously reported studies on *B. subtilis* mutants: all mutants with altered NAD^+ synthetase activity were shown to be severely affected in cellular functionality (5).

The recent resurgence of drug resistance tuberculosis poses a serious threat to the control of the desease and has caused much public concern (4). It has been demonstrated that a lack of nicotinic acid phosphoribosyltransferase activity in *Mycobacterium tuberculosis* blocks the NAD^+ recycling (7). Moreover, the use of the frontline antitubercular drug isoniazid may deplete pools of NAD^+: there is a decrease in NAD^+ of tubercule bacilli grown in the presence of isoniazid (3). These observations make the de novo NAD^+ biosynthesis in general and NAD^+ synthetase in particular possible targets for the development of new drugs against this microorganism.

Here we demonstrate that the *MTCY428.08* gene from *M. tuberculosis* is the structural gene for NAD^+ synthetase.

The *MTCY428.08* gene of *M. tuberculosis* is the structural gene of NAD^+ synthetase. The use of the *B. subtilis* NAD^+ synthetase sequence as a search probe against the sequenced *M. tuberculosis* genome showed significant homology with the *MTCY428.08* gene, coding for a polypeptide chain 738 residues long. The corresponding amino acid sequence compared with nonredundant databases at the National Center for Biotechnology Information by using the BLAST programs (1, 2) revealed low but significant homology with several NAD^+ synthetases. The highest similarity (P, $2.1e^{-21}$) was with the corresponding protein from *Rhodobacter capsulatus* (19). The degrees of sequence identity between the *M. tuberculosis MTCY428.08* gene product and NAD^+ synthetases from *R. capsulatus* and a *Synechocystis* sp. are, respectively, 31.4% (in a 191-residue overlap) and 29.7% (in a 182-residues overlap). The deduced amino acid sequence of the *MTCY428.08* gene was aligned, by using the CLUSTAL program (8), with those of other bacterial NAD^+ synthetases, revealing a strict

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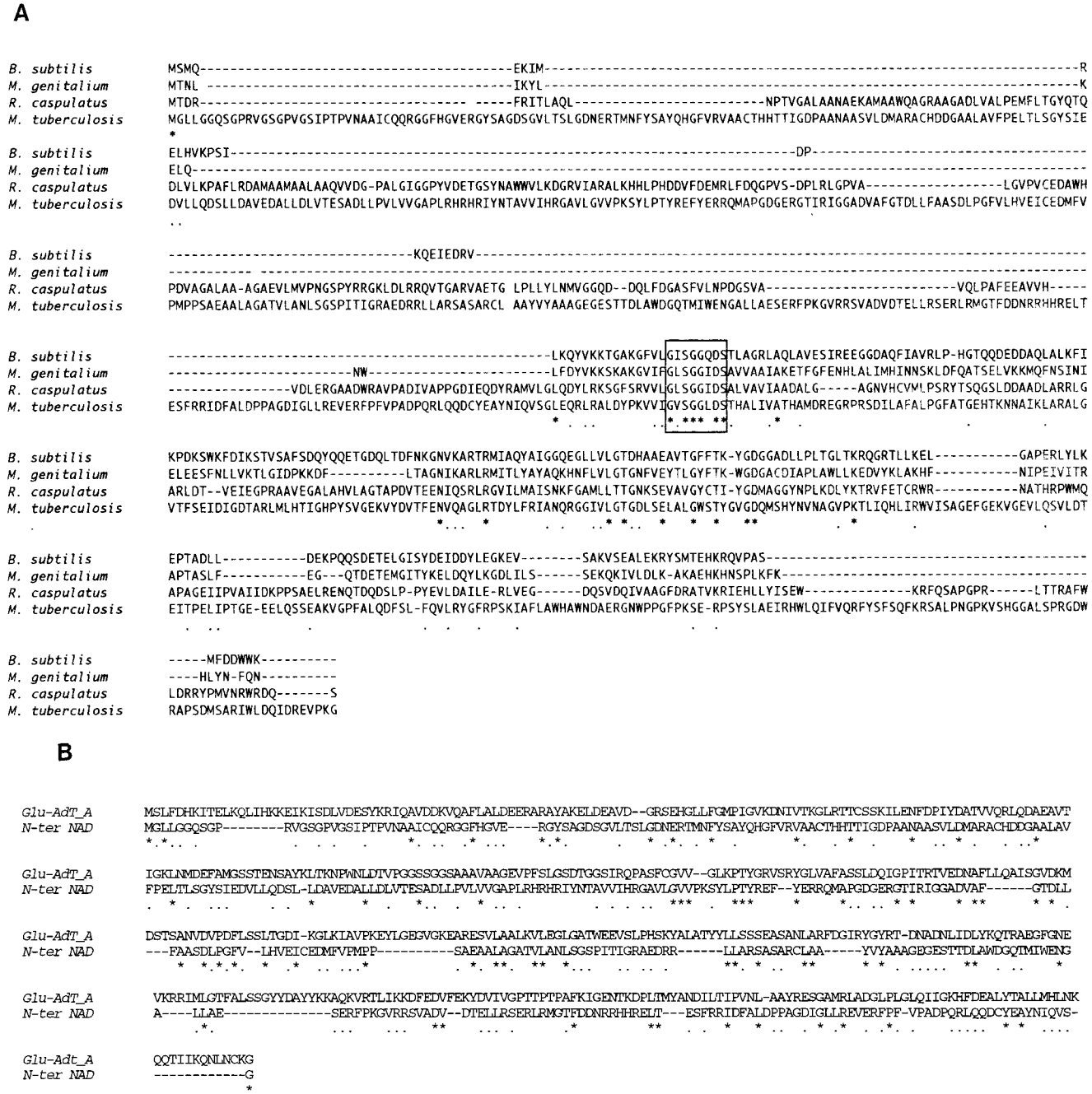

FIG. 1. (A) Amino acid sequence alignment of the *B. subtilis*, *M. genitalium*, *R. capsulatus*, and *M. tuberculosis* NAD^+ synthetases. The consensus fingerprint sequence for the N-type ATP-PPases (16) is boxed. Identical residues are indicated (asterisks). (B) Amino acids sequence alignment (8) of the A subunit of the *B. subtilis* Glu-AdT and the N-terminal (N-ter) region of the *M. tuberculosis* NAD synthetase.

conservation of residues showed to be important in catalysis (Fig. 1) (12). The MTCY428.08 amino acid sequence contains the fingerprint sequence SGGXDST (residues 427 to 433) proposed to be characteristic of a new family of ATP-PPases which includes NAD^+ synthetase (12, 16).

The *M. tuberculosis MTCY428.08* gene was cloned from cosmid MTCY428 (11) by PCR, according to standard protocol, into the expression vectors pGEX-4T-1 (14) and pET-15b (Novagen). The resulting plasmids were designated pCAB1 and pBAC2, respectively. pCAB1 and pBAC2 were transformed, by electroporation, into *Escherichia coli* JM105 and *E. coli* BL21(DE3), respectively, according to the supplier's instructions.

Following aerobic growth on 2× YTG medium (Pharmacia-Biotech) in the presence of ampicillin (100 μg/ml), total cell lysates of *E. coli* JM105, containing plasmid pGEX-4T-1 or pCAB1 (the recombinant plasmid), were prepared 120 min after the start of isopropyl-1-thio-β-D-galactopyranoside (IPTG) induction and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). *E. coli* JM105 cells

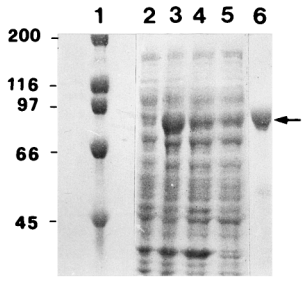

FIG. 2. SDS-PAGE analysis of the *M. tuberculosis* NAD synthetase purification profile. Lanes: 1, molecular weight standards; 2, *E. coli* BL21(DE3)(pET-15b) lysate supernatant; 3, *E. coli* BL21(DE3)(pBAC2) S-30 supernatant; 4, *E. coli* BL21(DE3)(pBAC2) S-100 supernatant; 5, *E. coli* BL21(DE3)(pBAC2) ultracentrifuged supernatant; 6, partially purified NAD^+ synthetase (arrow).

transformed with the pGEX-4T-1 vector showed high-level expression of the 26-kDa glutatione S-transferase (GST) protein. The presence of a 98-kDa polypeptide (the predicted molecular mass of the *M. tuberculosis* MTCY428.08 protein fused to GST) was clearly evident in extracts of *E. coli* JM105 cells harboring plasmid pCAB1 (data not shown). After aerobic growth on Luria-Bertani (LB) medium (13) in the presence of carbenicillin (200 μg/ml), total cell lysates of *E. coli* BL21 (DE3), containing plasmid pET-15b or pBAC2 (the recombinant plasmid), were prepared at various times from the start of IPTG induction and analyzed by SDS-PAGE. Growth at 37°C, even if the recombinant protein was highly expressed, caused the target protein to accumulate mainly as inclusion bodies. In order to avoid this undesirable effect, the incubation was carried out at 22°C without IPTG induction (Fig. 2, lane 2). In the latter case the His_6-tagged protein was purified through affinity chromatography on a Ni chelate resin according to the supplier's instructions (Fig. 2, lane 6). Care was taken to avoid oxidative conditions, maintaining a stable concentration of β-mercaptoethanol (10 mM) through all the purification steps. The first 30 N-terminal residues of the *MTCY428.08* gene product for both the GST-fused and His_6-tagged proteins were sequenced, confirming the expected sequence.

The activity of the *M. tuberculosis* NAD^+ synthetase enzyme was routinely assayed with ammonia or glutamine as nitrogen source as previously described (9, 21). NAD^+ synthetase activity was assayed on crude extracts of *E. coli* JM105 transformed with plasmids pGEX-4T-1 and pCAB1 or *E. coli* BL21 (DE3) transformed with plasmids pET-15b and pBAC2 and on the partially purified protein. The activity was expressed as nanomoles of NAD^+ synthesized per minute per milligram of protein. The results obtained are listed in Table 1.

The identification of the *M. tuberculosis* NAD^+ synthetase was firstly made possible through sequence comparison with other NAD^+ synthetases, at the National Center for Biotechnology Information. Sequence alignment revealed the strictly conserved sequence fingerprint characteristic of the N-type ATP-PPase (16). Moreover, a careful sequence comparison of the *B. subtilis* and *M. tuberculosis* enzymes, in light of the three-dimensional structure available for the former, showed a remarkable conservation of all the catalytically relevant residues (12).

TABLE 1. Activity of the *M. tuberculosis* NAD^+ synthetase expressed in *E. coli*

Strain	NAD^+ synthetase activity (nmol/min/mg of protein)	
	NH_3 dependent	Glutamine dependent
JM105(pGEX-4-T1)	0.28	0.28
JM105(pCAB1)	4.06	3.28
BL21(DE3)(pET-15b)	6.19	9.6
BL21(DE3)(pBAC2)	31.93	28.3
His_6 tag purified protein	230	90

The definitive confirmation that the *MTCY428.08* gene codes for NAD^+ synthetase was provided by determination of the enzymatic activity. When the dialyzed crude extracts of strain JM105(pCAB1) were assayed, the specific ammonia- or glutamine-dependent activities were found to be similar and roughly 15-fold greater than that of the control strain. The same behavior was detected for the BL21(DE3)(pBAC2) strain, but the specific activities were only six times greater than those of the control strain BL21(DE3)(pET-15b). These low values, even if undoubtedly confirming the presence of NAD^+ synthetase activity, may indicate that the enzyme is poorly expressed in its active form. After partial purification, the specific activity increased to a value of 230 nmol min^{-1} mg of $protein^{-1}$.

Another aspect clearly evidenced by the sequence alignment shown in Fig. 1 is a striking difference in molecular dimensions for the different NAD^+ synthetases, ranging from about 200 residues to 738 amino acids in the case of *M. tuberculosis* protein. A possible explanation of this fact is that the smaller enzymes represent only the synthetase subunit of the entire amidotransferase where the GAT domain is most likely synthesized as an independent subunit, whereas the bigger enzymes contain both domains on the same polypeptide chain. In this respect the observation that the *B. subtilis* enzyme (consisting of 271 residues) is strictly ammonia dependent (10) whereas the *M. tuberculosis* enzyme (consisting of 738 residues) is able to use ammonia or glutamine as nitrogen source with comparable specific activities (this study) is in keeping with our proposal. Recently an extensive study on *B. subtilis* Glu-$tRNA^{Gln}$ amidotransferase (Glu-AdT) was reported. Glu-AdT is a heterotrimeric protein whose A subunit, responsible for the amidotransferase function in this complex enzyme, does not have any obvious sequence relationship either with F-type or G-type known amidotransferases. On the other hand, it has a peculiar signature sequence, strictly conserved among a family of amidases. Therefore, it has been proposed to have a new type of amidase-like domain (6).

Interestingly, we observed significant sequence homology between the N-terminal region (residues 1 to 409) of the *M. tuberculosis* NAD synthetase and the Glu-AdT A subunit (Fig. 1B). However, the N-terminal domain of NAD synthetase does not possess the signature sequence, shared by Glu-AdT and several amidases (6). In this respect our finding suggests that the N-terminal region of NAD synthetase and the Glu-AdT A subunit may represent two members of a new type of GAT domain.

Our work provides the bases for future extensive characterization of this important enzyme from *M. tuberculosis*, hopefully including a crystallographic investigation, with the final aim of development of new antitubercular drugs.

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