A Eubacterial *Mycobacterium tuberculosis* tRNA Synthetase Is Eukaryote-like and Resistant to a Eubacterial-Specific Antisynthetase Drug†

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ABSTRACT: We report here the cloning and primary structure of *Mycobacterium tuberculosis* isoleucyl-tRNA synthetase. The predicted 1035-amino acid protein is significantly more similar in sequence to eukaryote cytoplasmic than to other eubacterial isoleucyl-tRNA synthetases. This similarity correlates with the enzyme being resistant to pseudomonic acid A, a potent inhibitor of *Escherichia coli* and other eubacterial isoleucyl-tRNA synthetases, but not of eukaryote cytoplasmic enzymes. Consistent with its eukaryote-like features, and unlike *E. coli* isoleucyl-tRNA synthetase, the *M. tuberculosis* enzyme charged yeast isoleucine tRNA. In spite of these eukaryote-like features, *M. tuberculosis* isoleucyl-tRNA synthetase exhibited highly specific cross-species aminoacylation, as demonstrated by its ability to complement isoleucyl-tRNA synthetase-deficient mutants of *E. coli*. When introduced into a pseudomonic acid-sensitive wild-type strain of *E. coli*, the *M. tuberculosis* enzyme conferred trans-dominant resistance to the drug. The results demonstrate that the sequence of a tRNA synthetase could have predictive value with respect to the interaction of that synthetase with a specific inhibitor. The results also demonstrate that mobilization of a pathogen's gene for a drug-resistant protein target can spread resistance to other, normally drug-sensitive pathogens infecting the same host.

Aminoacyl-tRNA synthetases play an essential function in all organisms by establishing the amino acid/trinucleotide algorithm of the genetic code through the aminoacylation of transfer RNAs (Carter, 1993; Lapointe & Giegé, 1991; Schimmel, 1987). Because of their role in fixing the relationship between nucleotide triplets and amino acids, the synthetases are believed to be ancient proteins whose evolutionary relationships provide insights into the development and speciation of life forms (Doolittle & Brown, 1994; Nagel & Doolittle, 1995; Schimmel & Henderson, 1994). At the same time, as essential components of the translation apparatus, the enzymes are potential targets for inhibitors that act as anti-infective drugs, provided that the fine structure of the active site of a particular synthetase varies enough for a drug to discriminate a pathogen synthetase from its human counterpart. While the catalytic domains of the 20 enzymes are divided into two basic classes (Eriani et al., 1990; Moras, 1992), and while the class to which a particular synthetase belongs is fixed in evolution, sequences within the catalytic domain of an enzyme in a given class can vary from prokaryotes to higher eukaryotes by more than 70% (Nagel & Doolittle, 1991). This variation provides ample opportunity to have species-specific inhibitors of any given tRNA synthetase.

Pseudomonic acid is the most prominent example of a species-specific inhibitor of a tRNA synthetase that has been developed as an anti-infective drug (Sutherland et al., 1985; Ward & Campoli-Richards, 1986). This compound is secreted by *Pseudomonas fluorescens* (Fuller et al., 1971) and acts as a potent inhibitor of many Gram-positive and -negative bacterial isoleucyl-tRNA synthetases. It has little or no activity on isoleucyl-tRNA synthetases from eukaryotes including *Homo sapiens* (Hughes & Mellows, 1980; data not shown). Although all isoleucyl-tRNA synthetase enzymes have the nucleotide-binding fold sequence motifs characteristic of class I tRNA synthetases (Hountondji et al., 1986; Webster et al., 1984), they can be subdivided into two types along eukaryote/eubacterial lines (Shiba & Schimmel, 1992b; Shiba et al., 1994b). For example, type 1 enzymes have specific shared sequence elements and a Cys_4 cluster at the C-terminus that forms a zinc-binding site that is essential for activity (Landro & Schimmel, 1994; Glasfeld and Schimmel, personal communication). Type 1 enzymes are found in eubacteria and eukaryote mitochondria. Type 2 enzymes are found in eukaryote cytoplasm. They lack the sequence elements specific to the type 1 enzymes, including the Cys_4 cluster, but have other sequence features that are peculiar to the type 2 isoleucyl-tRNA synthetases. Thus, the correlation of the sensitivity or resistance to pseudomonic acid with, respectively, the eubacterial or eukaryotic origin of the synthetase is possibly a reflection of there being an isoleucyl-tRNA synthetase in eukaryotes that is of a different type than those typically found in eubacteria.

Little is known about tRNA synthetases from mycobacteria such as *Mycobacterium bovis*, *Mycobacterium smegmatis*, and *Mycobacterium tuberculosis*. These are Gram-positive eubacterial organisms that are in the cluster composed of Actinomycetes in the universal phylogenetic tree (Dams et al., 1995). We assumed that, by virtue of being from a

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eubacterial organism, mycobacterial isoleucyl-tRNA synthetase would fall into the type 1 category and, for that reason, would be susceptible to pseudomonic acid. With this in mind, we cloned isoleucyl-tRNA synthetase from *M. tuberculosis*, determined its entire sequence, and expressed and characterized the protein. The results obtained were different from those expected and have several interesting ramifications, suggesting that *M. tuberculosis* isoleucyl-tRNA synthetase has an evolutionary history distinct from that of other eubacterial isoleucyl-tRNA synthetases.

MATERIALS AND METHODS

Materials. *M. tuberculosis* and *M. bovis* (BCG) genomic DNA were obtained from Drs. P. Brennan and J. Belize (Colorado State University, Fort Collins, CO). The *M. tuberculosis* λgt11 expression library was obtained from Professor R. Young (Whitehead Institute, Cambridge, MA). All oligonucleotides were synthesized on a Milligen Expedite 8909 DNA synthesizer. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Radionucleotides were purchased from Dupont NEN (Boston, MA). DNA for sequencing was isolated with the Wizard plasmid preparation kit from Promega (Madison, WI). Sequencing on both strands was done by using the dideoxy chain termination method (Sanger et al., 1977) in the presence of 10% dimethyl sulfoxide (DMSO) using the Sequenase kit from USB (Cleveland, OH) with the modified base 7-deazaguanosine. Sequence database searches were completed using the BLAST algorithm (Altschul et al., 1990). Protein and DNA sequence alignments and analysis were carried out by using programs from the Genetics Computer Group package (Madison, WI) and DNASTAR (Madison, WI). Southern blot transfer and other standard molecular biology techniques were done as described (Sambrook et al., 1989). *E. coli* strain DH5α (Hanahan, 1983) was used for plasmid propagation. Protein concentrations were determined by using Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Pseudomonic acid A was a gift from Abbott Laboratories, IL.

PCR Amplification of Isoleucyl-tRNA Synthetase from Mycobacterium kansasii DNA. The degenerate oligonucleotide primers used for the PCR cloning of *Mycobacterium* isoleucyl-tRNA synthetase are the same as those used previously (Shiba et al., 1994b). Combinations of the various primers were tested in PCR reactions containing 10 ng of *M. kansasii* DNA template per 50 μL reaction. PCR reactions, DNA purification, cloning of PCR fragments, and identification of inserts were done as described by Shiba et al. (1994b).

Screening of the M. tuberculosis Expression Library. Two DNA probes from *M. kansasii* genomic DNA corresponding to the 5′-end (379 bp) and 3′-end (714 bp) of the largest PCR fragment (1487 bp) were generated by PCR using the following specific primer combinations: 5′-TCACCGACAAATCGCAGATCGACGCCAT-3′/5′-AATGCGCCGGCCAGCTCTTCAA-3′ and 5′-GGCGTACGGCGAAGACGACATGGCGG-3′/5′- GGATCGGCGCAATCGACCCCGT-3′. They were then purified using the GeneClean II kit (Bio101, Vista, CA) and radiolabeled by nick translation (Boehringer-Mannheim, Indianapolis, IN), with unincorporated nucleotides being removed by gel filtration using prepacked Nap-5 columns (Pharmacia, Piscataway, NJ). The radiolabeled DNAs were used to screen duplicate nylon membranes (GeneScreen Plus, Dupont, Boston, MA) containing 20000−30000 pfu from the *M. tuberculosis* λgt11 library. *E. coli* strain Y1090 (Young & Davis, 1983) was used for phage infection. Prehybridization and hybridization solutions consisted of 5× SSC/5× Denhardt's (0.1% of each of bovine serum albumin, poly(vinylpyrrolidone) and Ficoll Type 400)/0.5% sodium dodecyl sulfate (SDS)/5 mM EDTA. Each filter was incubated overnight at 65 °C with 10^6 cpm of probe per milliliter of hybridization solution, washed in 2× SSC/0.1% SDS at 65 °C, and exposed to X-ray film (Kodak X-OMAT). Eight plaques hybridizing to both probes were purified for further characterization. The 5′- and 3′-ends of the gene were each mapped to two overlapping clones MS3 and SS5B, containing 5 and 2 kb inserts, respectively.

Plasmids for the Expression of M. tuberculosis Isoleucyl-tRNA Synthetase in E. coli. To facilitate subcloning the inserts from the λgt11 clones, we constructed two vectors pBSi and pNBS by introducing a *Bsi*WI site and *Spe*I, *Bsi*WI, and *Not*I sites, respectively, into pUC19 (Yanisch-Perron et al., 1985) by using standard protocols.

Vectors pUC19 and pBSi were used to subclone fragments MS3 and SS5B isolated from the λgt11 clones, respectively, to yield pMS3 (containing the 5′-end of the gene) and pSS5B (containing the 3′-end of the gene). The full isoleucyl-tRNA synthetase gene was reconstructed in pNBS (pNBS-Ile) from these two overlapping subclones by using an internal *Bam*HI site common to both clones.

E. coli Expression Constructs. To facilitate expression in *E. coli*, the G of the GTG initiation codon was modified to an A by PCR mutagenesis to yield an ATG initiation codon (in bold) using the oligomer 5′-ATAAGAATGCGGCCGCAGATCT**ATG**ACCGATAACGCATATCCAA-3′. A *Bgl*II site (underlined) was introduced upstream of the ATG to allow cloning into the *Bam*HI site of the expression vectors.

To construct plasmid pKS56, the modified 5′-end and the 3′-end of the *IleRS* gene were ligated to *Bam*HI/*Xba*I-digested pBSKS vector (Stratagene, La Jolla, CA), so that the *M. tuberculosis* isoleucyl-tRNA synthetase gene is put under the control of the lac promoter to express a native protein. To construct pGX56, the modified 5′-end and the 3′-end of the *IleRS* gene were ligated into *Bam*HI/*Sma*I-digested pGEX-4T-2 (Pharmacia) to yield a fusion construct in which the 5′-end of the isoleucyl-tRNA synthetase gene is fused in-frame with the *GST* gene. To construct pTR56, which expresses the native *M. tuberculosis* isoleucyl-tRNA synthetase under the control of the lacUV5 promoter, the modified 5′-end and the 3′-end of the *IleRS* gene were ligated into pTrc99A (Pharmacia). All plasmids were partially sequenced to confirm their structures.

Complementation in E. coli. The MI1′ strain (Starzyk et al., 1987) is the RecA$^-$ derivative (*recA*::*Tn*10) of the original MI1 strain (*ileS1 argH trpA36*) (Iaccarino & Berg, 1970) containing a mutation in the *ileS* gene. For complementation of isoleucine auxotrophy, MI1′ cells were transformed with plasmids pTR56, pKS56, or pGX56, *E. coli ileS*-containing pKS21 (Shiba & Schimmel, 1992a), and control vectors pBSKS and pGEX-4T-2. Transformants were selected on LB−ampicillin (LB−amp) plates (60 μg/mL) at 37 °C. Six individual colonies from each transformation were transferred onto minimal M9−amp agar plates containing 50 μM IPTG

and supplemented with thiamine, tryptophan, and arginine or onto LB−amp plates.

For complementation of temperature-sensitive *E. coli* null mutants, the test construct (pTR56), positive control construct (pKS21), and vector alone (pBSKS) were introduced into *E. coli* strain IQ844/pRMS711 at 30 °C (Shiba & Schimmel, 1992b). For each construct, growth of transformants was scored on M9 minimal plates containing 0.2% casamino acids at 37 or 42 °C. Colonies that grew on these plates were tested for chloramphenicol sensitivity to verify the loss of the *ileS* maintenance plasmid, as described (Shiba & Schimmel, 1992b).

Purification of the GST−IleRS Fusion Protein. E. coli strain JM109 containing plasmid pGX56 was grown in LB−amp (100 μg/mL) at 37 °C to midlog phase. IPTG was added to 1 mM, and the incubation temperature was shifted to 18 °C with vigorous shaking for 72 h. The cells were harvested by centrifugation (6000 rpm for 10 min at 4 °C) in a Sorvall SL-250T rotor. Cell pellets were resuspended in lysis buffer (50 mM potassium phosphate (pH 7.5), 0.1 M NaCl) containing 10 mM dithiothreitol (DTT). The cells were lysed using a French press, and the lysate was clarified by centrifugation at 20000*g* for 30 min in a Sorvall SL-50T. The clarified extract was loaded onto a glutathione-Sepharose 4B column (Pharmacia) equilibrated with PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) containing 2 mM DTT. Bound protein was eluted with 10 mM reduced glutathione in 50 mM Tris (pH 7.5) and concentrated in a C-10 Centricon (Amicon, Beverly, MA). Thrombin cleavage was carried out at room temperature in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5 mM $CaCl_2$, and 2 mM DTT with 0.5 unit of thrombin (Sigma, St. Louis, MO) for 16 h.

Aminoacylation Assays. Clarified lysate or purified proteins were tested for aminoacylation activity in a heat block at 25 °C, according to Shiba and Schimmel (1992b). Specifically, enzyme was added to a reaction mixture (50−100 μL final volume) containing 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.5), 8 mM $MgCl_2$, 20 μM L-[4,5-^{3}H]isoleucine (2200 cpm/pmol, New England Nuclear), 10 mM KF, 4 mM ATP, and 90 μM total tRNA from either *E. coli* (Sigma) or brewer's yeast (Boehringer-Mannheim). At various time intervals, 10−20 μL aliquots were withdrawn, precipitated with cold TCA (5%) onto Whatman 3MM cellulose filter paper, and treated as described (Shiba & Schimmel, 1992b). The amount of [^{3}H]isoleucine incorporated into tRNA bound to the filter was determined by scintillation counting in 5 mL of Betafluor (Packard 1600 TR scintillation counter). K_m values were determined for the purified GST−IleRS fusion protein with and without thrombin cleavage in the presence of either 200 μM total yeast tRNA or 300 μM total *E. coli* tRNA in a reaction volume of 50 μL. Isoleucine concentrations were varied from 0.25 to 32 μM, while the enzyme concentration was 6.5 nM.

PMA Rescue. E. coli strains DH5α and Novablue (Novagen) were transformed with pGEX-4T-2, pKS21, pKS56, and pGX56. Transformants were grown in LB−amp (100 μg/mL) overnight at 37 °C, diluted 1:20 into LB−amp containing 25 μM IPTG, grown for 5−8 h at 30 °C, and then diluted in LB−amp containing 25 μM IPTG to an of 0.1. An aliquot (10 μL) from each culture was placed in a 96-well microtiter plate, to which was added 100 μL of medium (LB−amp containing 25 μM IPTG with varying amounts of pseudomonic acid). The plate was incubated at 30 °C (shaking at 200 rpm) for 36−40 h, and A_{600} values were read on a SpectraMax 250 plate reader (Molecular Devices, Sunnyvale, CA).

RESULTS

Cloning the M. tuberculosis Gene Encoding Isoleucyl-tRNA Synthetase. Alignment of sequences of isoleucyl-tRNA synthetase from several organisms identified discrete conserved regions that were used previously to design degenerate primers for cross-species PCR cloning of fragments of these genes (Shiba et al., 1994b). In this work, five out of eight combinations of previously described degenerate primers (Shiba et al., 1994b) yielded PCR products of the expected size when *M. kansasii* genomic DNA was used as a template. Sequencing of these fragments showed a high GC content (≥62%), a feature that is characteristic of mycobacterial DNA (Hatfull & Sarkis, 1993). Searches of GenBank and the Swiss Protein Bank showed that the translated PCR fragments were homologous to other known sequences of isoleucyl-tRNA synthetases.

The ^{32}P-radiolabeled *M. kansasii* PCR products cross-hybridized at high stringency to a single band of approximately 3.6 kb in *Sma*I-digested *M. tuberculosis* and *M. bovis* DNA (data not shown), indicating a high DNA sequence homology among isoleucyl-tRNA synthetase genes from various mycobacteria. These same fragments did not hybridize to *E. coli* DNA. We concluded that the gene for *M. tuberculosis* isoleucyl-tRNA synthetase could be isolated by screening a *M. tuberculosis* genomic library with *M. kansasii* PCR fragments as probes.

On the basis of the sequence of the largest *M. kansasii* PCR fragment (1.5 kb), two specific probes were generated and used to screen a λgt11 expression library of *M. tuberculosis* (Young et al., 1985). Eight out of 50 000 plaques hybridized with both probes and DNA samples were isolated from purified plaques for further characterization. Restriction enzyme digests indicated the presence of six different insert populations of *M. tuberculosis* DNA. The 3′- and 5′-ends of the gene were mapped by Southern hybridization (Southern, 1975) to two overlapping clones, whose inserts were subcloned and sequenced to identify the entire *M. tuberculosis* isoleucyl-tRNA synthetase gene and its flanking regions.

An open reading frame was identified with two possible GTG start sites (Figure 1). GTG (GUG in the mRNA) initiation codons have been observed in *Mycobacterium* genes, as well as in other GC-rich organisms (Hatfull & Sarkis, 1993). However, where a codon other than AUG observed, invariably the amino acid used for initiation was determined to be methionine (Varshney et al., 1991). For that reason, we assumed that the N-terminal GUG acts as the initiator and codes for methionine. On the basis of alignments with sequences of other isoleucyl-tRNA synthetases, we tentatively assigned the second GTG as the initiation codon.

A schematic diagram of the 1035-amino acid (≈120 kDa) protein is shown in Figure 1. As in other class I synthetases, the nucleotide-binding fold of the *M. tuberculosis* isoleucyl-tRNA synthetase is located in the N-terminal half of the protein. We have assigned elements of secondary structure

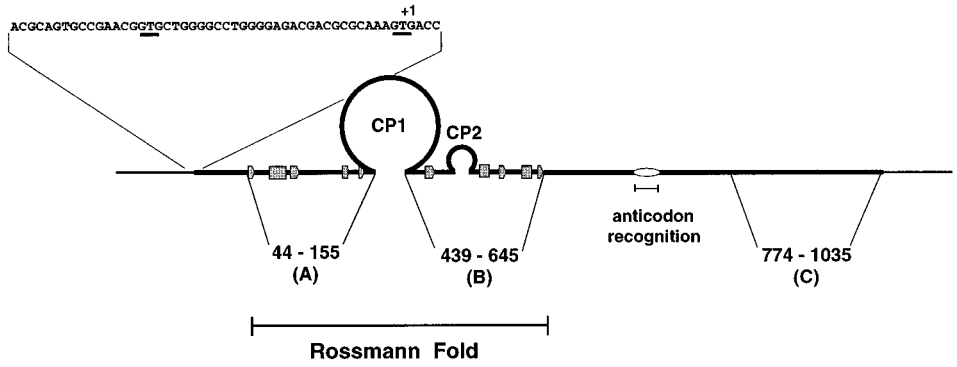


FIGURE 1: Schematic representation of *M. tuberculosis* isoleucyl-tRNA synthetase. The thick line represents the open reading frame. The 5′- and 3′-untranslated regions flanking the gene are indicated arbitrarily by the thin line. The DNA sequence at the 5′-end of the gene is shown, and the two possible in-frame GTG start sites are underlined. Initiation of translation at the second GTG codon produces a protein of 1035 amino acids. The enzyme encoded by the gene has two major domains, of which the N-terminal class I-defining catalytic domain (residues 1−645) that contains the Rossmann fold and the connective peptides CP1 and CP2 is shown in more detail. Gray rectangles represent α-helices while gray pentagons represent β-strands. Regions A−C were chosen for multiple alignments shown in Figure 2.

according to Starzyk et al. (1987) and Shiba and Schimmel (1992a), who used the three-dimensional structure of the closely related *E. coli* methionyl-tRNA synthetase (Brunie et al., 1987, 1990) as a guide. The fold is made up of alternating β-strands and α-helices in a $\beta_6\alpha_4$ arrangement that is divided into two $\beta_3\alpha_2$ halves. In the first half (Figure 2A), the 11-amino acid signature sequence, which ends in the HIGH tetrapeptide (Webster et al., 1984), is located in a loop between the first β-strand and α-helix. In the second half (Figure 2B), the KMSKS pentapeptide (Hountondji et al., 1986) is located in a loop between the second β-strand and α-helix. These sequence elements are characteristic landmarks of class I enzymes.

The two halves of the nucleotide-binding fold are divided by an insertion known as connective polypeptide 1 (CP1) (Starzyk et al., 1987). In the cocrystal of the class I glutaminyl-tRNA synthetase with $tRNA^{Gln}$, this insertion provides for synthetase interactions with the acceptor helix (Rould et al., 1989). The sequence of this 284-amino acid insertion is not shown in Figure 2. A second insertion (CP2) is located in the second half of the nucleotide-binding fold between its first β-strand and α-helix. The function of this insertion, which is not shared by all class I enzymes, is not known.

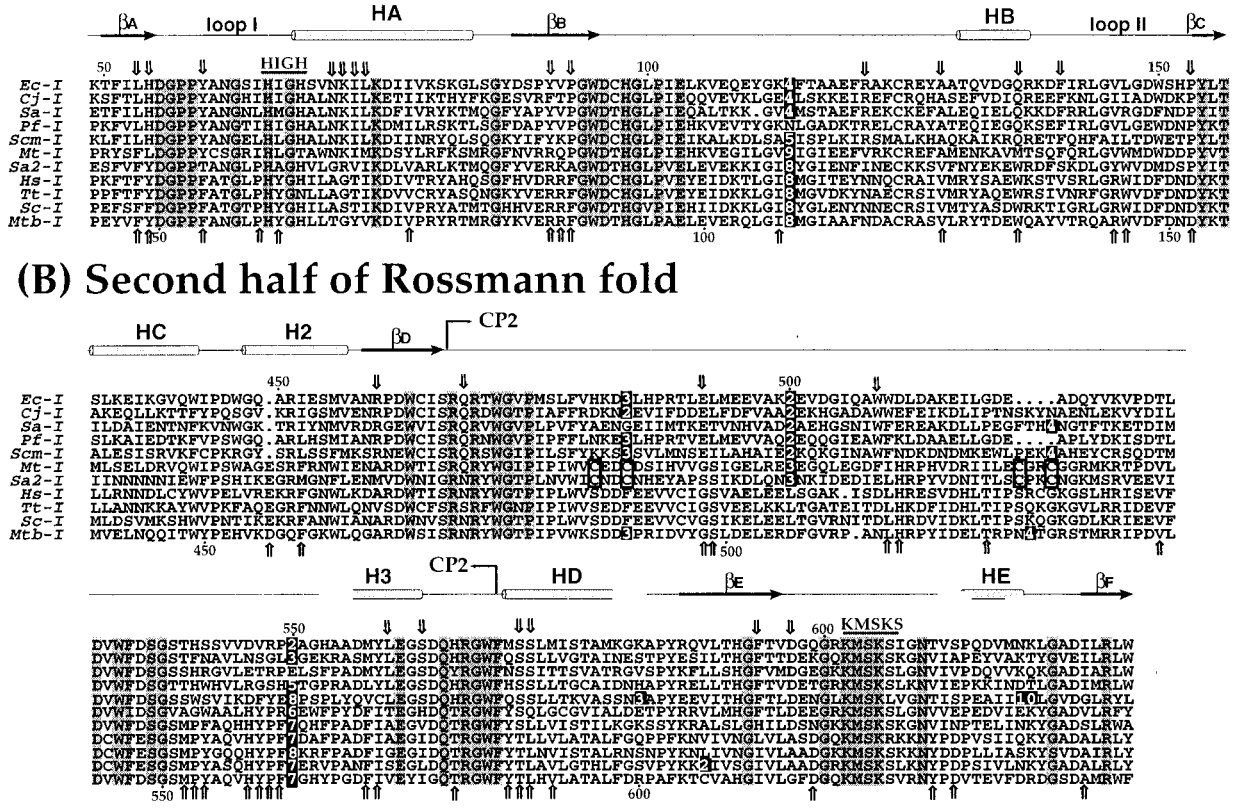
The nucleotide-binding fold extends through F645 of the *M. tuberculosis* enzyme. The remaining 390 amino acids make up the C-terminal domain, which predominantly comprises α-helices. This domain has determinants for interactions with the anticodon of $tRNA^{Ile}$ (Auld & Schimmel, 1995; Shepard et al., 1992). Only the last 261 amino acids are shown in the alignment in Figure 2C.

The Sequence of M. tuberculosis Isoleucyl-tRNA Synthetase Appears Eukaryote-like. Surprisingly, the sequence of *M. tuberculosis* isoleucyl-tRNA synthetase aligned most closely with sequences of eukaryotic isoleucyl-tRNA synthetases, having between 38 and 43% overall sequence identity, depending on the particular pairwise comparison (Figure 2). In contrast, the overall percent identity to sequences of eubacterial isoleucyl-tRNA synthetases was between 20 and 28%. Most striking is the absence in the C-terminus of the *M. tuberculosis* enzyme of the cysteine cluster, which forms a characteristic zinc-binding motif in eubacterial isoleucyl-tRNA synthetases (type 1) (Landro et al., 1994), suggesting that isoleucyl-tRNA synthetase from *M. tuberculosis* is more similar to the type 2 enzymes present in eukaryotes (Figure 2C). The enzyme from the archaebacterium *Methanobacterium thermoautotrophicum* (Jenal et al., 1991) and the seemingly related extrachromosomal isoleucyl-tRNA synthetase found in *Staphylococcus aureus* strains that are highly resistant to pseudomonic acid (Gilbart et al., 1993) also lack the C-terminal cysteine cluster, but in contrast to type 1 and type 2 enzymes, both have a cysteine cluster in CP2, suggesting a common ancestor for the *M. thermoautotrophicum* and extrachromosomal *S. aureus* enzymes (Figure 2B).

Amino acid residues and motifs in the *M. tuberculosis* protein that deviate from the eubacterial consensus and regions that contribute to its classification as a eukaryote-like protein are also shown in Figure 2. Specifically, the *M. tuberculosis* enzyme lacks the NKIL consensus amino acids downstream of the HIGH sequence, as well as other residues in each half of the Rossman fold that are conserved in the five known sequences from eubacteria. Critical positions where differences occur between the two types of enzyme are indicated by arrows. In addition, and in contrast to the eubacterial enzymes, the *M. tuberculosis* enzyme has the MPY_{554}, $HYPF_{561}$, FI_{576}, and $YTLXV_{592}$ motifs found only in the eukaryotic enzymes (numbering based on the *M. tuberculosis* protein).

Consistent with this observation, the phylogenetic tree that was constructed according to the method of Hein (1990) by using the alignment of the second half of the nucleotide-binding fold (including CP2) clearly indicates that the *M. tuberculosis* enzyme belongs to the eukaryotic type 2 enzymes (Figure 3). Comparable relationships were obtained when the phylogenetic analysis was done with the first half of the nucleotide-binding fold. Moreover, the branching pattern of the tree is similar to that obtained by using other programs, thus strengthening the validity of the calculation.

The *M. kansasii* isoleucyl-tRNA synthetase gene also appears to encode a type 2 enzyme: DNA sequence identity between *M. tuberculosis* and *M. kansasii* (spanning 1.5 kb) was over 82%, with most of the differences found at the third positions of the codons. As a consequence, protein sequence identity was over 87%. To determine whether the eukaryote-like features of the *M. tuberculosis* and *M. kansasii* enzymes were unique or indicative of all mycobacteria, we also isolated and sequenced part of the isoleucyl-tRNA synthetase gene from *M. smegmatis*, a nonpathogenic mycobacterium. The partial sequence from *M. smegmatis*



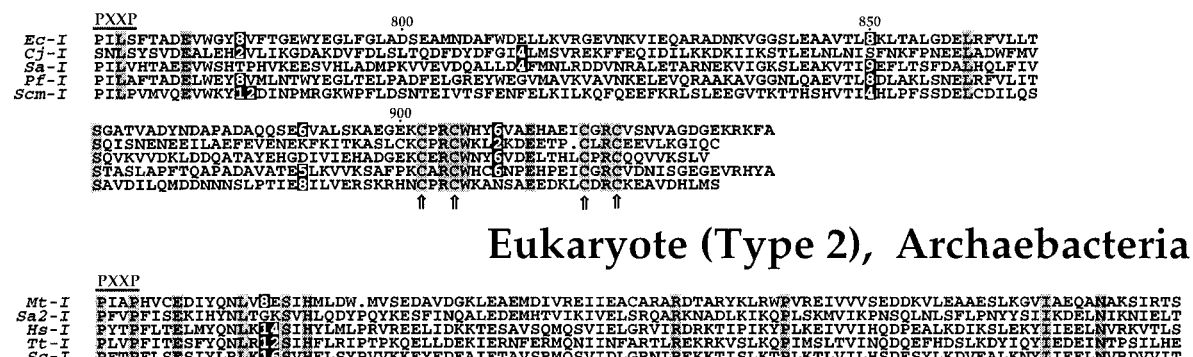


FIGURE 2: Alignment of portions of the *M. tuberculosis* isoleucyl-tRNA synthetase coding sequences with those of other isoleucyl-tRNA synthetases. The sequence is divided into three regions designated A−C. Regions A and B correspond to the two halves of the nucleotide-binding fold (Rossmann fold), which are separated by the CP1 insertion (not shown). Region C corresponds to the C-terminus of the protein and is divided between enzymes that have a terminal cysteine cluster (eubacteria type 1) and enzymes that lack the terminal cysteine cluster (eukaryotes type 2 and archaebacteria). Alignments were done using the PILEUP program in the GCG package (Madison, WI). Solid horizontal arrows represent β-strands and cylinders represent α-helices and are labeled according to Shiba et al. (1994b). Numbering at the top corresponds to the *E. coli* and at the bottom to the *M. tuberculosis* enzyme. The shaded areas show amino acid identities in all sequences. In (A) and (B), vertical arrows on the top line indicate positions at which the amino acids in the *M. tuberculosis* enzyme deviate from eubacterial species; vertical arrows on the bottom line indicate positions at which the *M. tuberculosis* enzyme follows the eukaryotic pattern. In (B), the cysteine clusters present only in CP2 of the Sa-2 and Mt enzymes are boxed. In (C), vertical arrows indicate the locations of the cysteine clusters in the eubacterial enzymes. Amino acid 827 (X) is either Leu (L) or Val (V) (sequencing of both strands did not resolve the ambiguity). The species abbreviations are as follows: *Escherichia coli* (Ec), *Campylobacter jejuni* (Cj), *Staphylococcus aureus* (Sa), *Pseudomonas fluorescens* (Pf), *Saccharomyces cerevisiae* mitochondria (Scm), *Methanobacterium thermoautotrophicum* (Mt), *Staphylococcus aureus* PMA^R plasmid-borne isoleucyl-tRNA synthetase (Sa2), *Homo sapiens* (Hs), *Tetrahymena thermophila* (Tt), and *Mycobacterium tuberculosis* (Mtb).

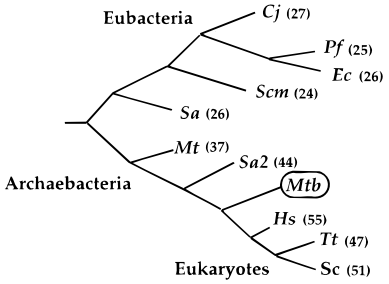


FIGURE 3: Phylogenetic tree based on sequences of isoleucyl-tRNA synthetases. On the basis of sequence alignments in region B (Figure 2), a phylogenetic tree was constructed by the method of Hein (1990). The length of each branch is proportional to the genetic distance. Numbers in parentheses represent similarities (percentage) between the isoleucyl-tRNA synthetase enzyme of *M. tuberculosis* and those of other organisms. The eukaryote-like sequence of the *M. tuberculosis* enzyme is clearly evident.

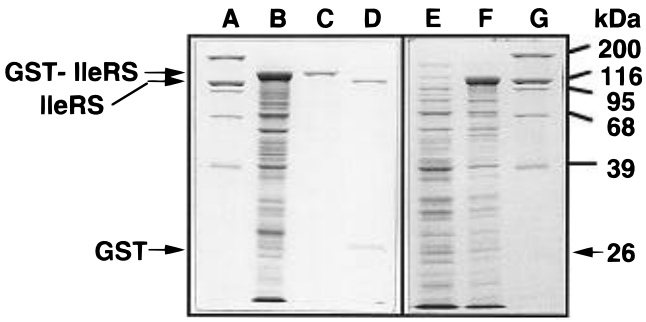


FIGURE 4: Expression of the *M. tuberculosis* isoleucyl-tRNA synthetase protein in *E. coli*. The Coomassie Blue stain of a 10% SDS−PAGE gel is shown. *E. coli* strain JM109 containing either plasmid pGX56 encoding a GST−isoleucyl-tRNA synthetase fusion protein (GST−IleRS) or plasmid pTR56 encoding native *M. tuberculosis* isoleucyl-tRNA synthetase (IleRS) was induced with 1 mM IPTG for 3 days at 18 °C. Lanes contain molecular weight markers (A and G), clarified JM109/pGX56 cellular extract (B), affinity-purified GST−IleRS (C), thrombin-cleaved GST−IleRS (D), and clarified JM109/pTR56 cellular extract without (E) or with (F) IPTG induction.

showed high homology to the respective *M. tuberculosis* region. We conclude that all three mycobacterial enzymes investigated here are type 2 (data not shown).

Expression of M. tuberculosis Isoleucyl-tRNA Synthetase in E. coli. To study the properties of the *M. tuberculosis* enzyme *in vitro* as well as *in vivo*, DNA constructs were made for expression in *E. coli*. The full-length isoleucyl-tRNA synthetase gene was first obtained by ligating DNA fragments containing the 5′- and 3′-ends of the gene into a pUC19-derived vector to generate plasmid pNBS-Ile. Further modifications for expression of the *M. tuberculosis* gene in *E. coli* were introduced by PCR mutagenesis (see Materials and Methods). The gene for *M. tuberculosis* isoleucyl-tRNA synthetase was cloned under the control of the *lac* or *tac* promoters to express either a native protein or an N-terminal glutathione *S*-transferase (GST) fusion protein, respectively. In these constructs, the 5′-end of the gene was modified by PCR mutagenesis, such that the GTG initiation codon was changed to ATG to optimize expression in *E. coli*. The constructs were introduced into *E. coli*, and the expressed proteins, as visualized by SDS−PAGE of crude extracts, were about 120 kDa for the native (pTR56) and 145 kDa for the GST fusion protein (pGX56) (Figure 4).

M. tuberculosis Isoleucyl-tRNA Synthetase Aminoacylates Total tRNA from Both E. coli and Yeast. Activity of the native and GST fusion proteins was investigated by using two different host cells for expression. First, the GST fusion protein (GST−IleRS, encoded by plasmid pGX56) was expressed in *E. coli* strain JM109 and affinity purified on a glutathione−agarose column. The purified GST−IleRS was active and efficiently aminoacylated total *E. coli* tRNA. The GST moiety was efficiently removed by thrombin cleavage at 16 °C (Figure 4). The K_M for isoleucine of the affinity-purified GST−IleRS was 1.1 μM at pH 7.4, 25 °C. [This K_m value is comparable to that (≈4 μM) for the *E. coli* enzyme (Baldwin & Berg, 1966; Fersht, 1977; Schmidt & Schimmel, 1994).] The K_m and rate of aminoacylation did not vary following the removal of the GST moiety, implying that the GST−IleRS fusion protein has the same enzymatic properties as those of the native protein.

Second, because of concern about contaminating activity from endogenous *E. coli* isoleucyl-tRNA synthetase, the activity of the *M. tuberculosis* unfused protein (encoded by plasmids pKS56 and pTR56) was tested in the *E. coli* isoleucine auxotroph strain MI1′ (Iaccarino & Berg, 1970; Schmidt & Schimmel, 1994). MI1′ cells carry a single point mutation in the *ileS* gene (F570S), resulting in an approximately 1750-fold increase in the K_m for isoleucine from 4 μM to 7 mM (Schmidt & Schimmel, 1994). As a result, the *in vitro* charging activity of MI1′ cellular extracts is negligible in assays containing 20 μM isoleucine. Extracts of MI1′ cells alone or transformed with the vector do not have significant charging activity, when tested under standard aminoacylation conditions with an isoleucine concentration of 20 μM. In contrast, crude extracts of MI1′ cells transformed with plasmids expressing native *M. tuberculosis* isoleucyl-tRNA synthetase contained an enzymatic activity comparable to that of the purified GST−IleRS fusion protein and were able to efficiently aminoacylate *E. coli* total tRNA with isoleucine (data not shown).

Because the *M. tuberculosis* enzyme resembles eukaryotic synthetases, we thought that, unlike all eubacterial isoleucyl-tRNA synthetase enzymes tested so far, the *M. tuberculosis* enzyme might charge yeast tRNA. We tested *in vitro* aminoacylation activities of both native and purified GST-fused *M. tuberculosis* isoleucyl-tRNA synthetase in the presence of total yeast tRNA. Both proteins were found to aminoacylate yeast tRNA efficiently (Figure 5 and data not shown). Partially purified isoleucyl-tRNA synthetase from *M. smegmatis* was also found to aminoacylate both yeast and *E. coli* tRNA (data not shown). In contrast, the purified *E. coli* enzyme showed no detectable aminoacylation activity with yeast tRNA as substrate (Figure 5).

Although total tRNA from both *E. coli* and yeast was efficiently aminoacylated by the *M. tuberculosis* enzyme, it was possible that noncognate tRNAs were being misaminoacylated. To test this hypothesis, total yeast tRNA was first incubated with purified *Candida albicans* isoleucyl-tRNA synthetase (Kaufmann, 1995) until all isoleucyl-tRNAs were aminoacylated with radioactive isoleucine. The purified *M. tuberculosis* (GST−IleRS) or *C. albicans* isoleucyl-tRNA synthetase was then added to determine whether additional tRNAs could be aminoacylated. Similarly, total *E. coli* tRNA was first incubated with *E. coli* isoleucyl-tRNA synthetase, and then *M. tuberculosis* enzyme was added. Upon the addition of purified GST−IleRS, no further aminoacylation was detected in either system, suggesting that the *M. tuberculosis* isoleucyl-tRNA synthetase is not sig-

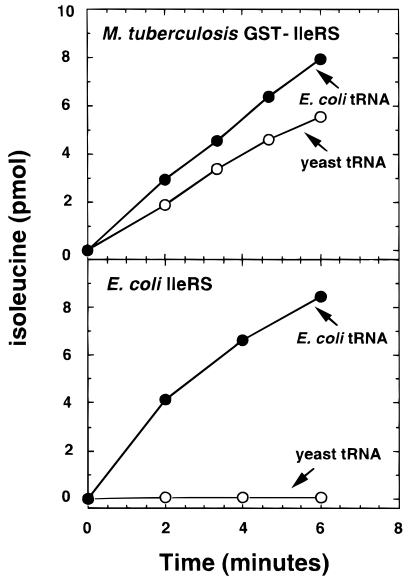


FIGURE 5: *In vitro* aminoacylation activities of purified *M. tuberculosis* and *E. coli* isoleucyl-tRNA synthetases. The *M. tuberculosis* enzyme aminoacylates tRNAs from both eubacteria (*E. coli)* and eukaryotic organisms (*S. cerevisiae)*. Charging assays were done at pH 7.5, 25 °C as described in Materials and Methods. Top panel: Charging activity of affinity-purified *M. tuberculosis* GST−isoleucyl-tRNA synthetase in the presence of 90 μM total yeast tRNA (open circles) or *E. coli* tRNA (closed circles). Bottom panel: Charging activity of purified *E. coli* isoleucyl-tRNA synthetase in the presence of 90 μM total yeast tRNA (open circles) or *E. coli* tRNA (closed circles) substrate. Final reaction volumes were 50 μL, containing *M. tuberculosis* GST fusion protein (13 nM) or *E. coli* protein (0.5 nM). The background (no tRNA control) was subtracted for each experiment.

nificantly misaminoacylating the tRNAs (data not shown). To establish a positive control and to confirm these results in a different way, total yeast tRNA was first incubated with either purified *E. coli* isoleucyl-tRNA synthetase or purified *C. albicans* tyrosyl-tRNA synthetase (P. Gallant and M. Sassanfar, unpublished results) in the presence of both tritium-labeled isoleucine and tyrosine until all tRNA substrates were exhausted. Purified *M. tuberculosis* GST−IleRS was then added, and the appearance of newly radiolabeled tRNAs was detected in both cases upon the addition of the *M. tuberculosis* enzyme.

M. tuberculosis Isoleucyl-tRNA Synthetase Can Substitute for E. coli Isoleucyl-tRNA Synthetase in Vivo. Because *M. tuberculosis* isoleucyl-tRNA synthetase efficiently charges *E. coli* tRNA, we investigated whether it could substitute entirely for the function of the *E. coli* enzyme in either an *E. coli ileS* null strain or the auxotrophic MI1′ strain. *E. coli* null strain IQ844/pRMS711 (Shiba & Schimmel, 1992b) contains a chromosomal deletion of the *ileS* gene. Growth of this strain is maintained by the wild-type copy of *ileS* that is carried on plasmid pRMS711, which has a temperature-sensitive replicon. Following the introduction of a plasmid encoding unfused *M. tuberculosis* enzyme (pTR56) or a plasmid containing wild-type *E. coli ileS* (pKS21) into strain IQ844/pRMS711 at 30 °C, growth of the transformants was scored on M9 medium containing casamino acids at either 37 or 42 °C, temperatures at which the maintenance plasmid pRMS711 cannot replicate. Transformants containing either pTR56 or pKS21 formed colonies on plates at the higher temperatures, whereas control cells did not form any colonies. After ejection of the maintenance plasmid, cells

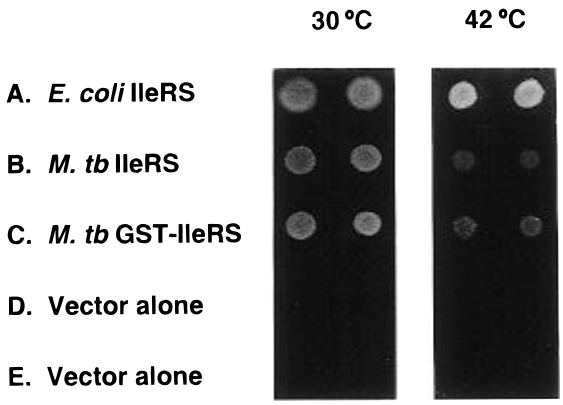


FIGURE 6: Complementation of *E. coli* MI1′ isoleucine auxotrophs by *M. tuberculosis* isoleucyl-tRNA synthetase. Single colonies of MI1′ cells transformed with (A) pKS21 (encoding *E. coli ileS*), (B) pKS56 (encoding *M. tuberculosis* isoleucyl-tRNA synthetase), and (C) pGX56 (encoding *M. tuberculosis* GST−isoleucyl-tRNA synthetase fusion protein) or vectors pBSKS (D) and pGEX-4T-2 (E) were transferred from LB−amp plates to minimal agar plates containing 100 μg/mL ampicillin. The plates were incubated at 30 or 42 °C for 48 h. The slow growth at 42 °C of MI1′ cells containing the *M. tuberculosis* enzyme is correlated to poor *in vitro* aminoacylation activity at that temperature.

containing pTR56 grew slightly slower than cells containing pKS21 at 25, 30, and 37 °C (data not shown). At 42 °C, the cells sustained by pTR56 showed impaired growth and could grow only on M9 medium containing casamino acids, but not on rich medium. The less robust growth at 42 °C could be explained by a 40% decrease in aminoacylation activity of the *M. tuberculosis* enzyme *in vitro* at 42 °C compared to 30 °C. On the contrary, *E. coli* isoleucyl-tRNA synthetase had little decrease (10%) in activity at 42 °C (data not shown).

Similar results were observed when the auxotrophic strain was used. MI1′ cells containing constructs expressing either unfused (pTR56) or GST−IleRS fusion protein (pGX56) grew well at 30 and 37 °C, but poorly at 42 °C on minimal medium, whereas cells containing the vectors alone did not grow on minimal medium unless supplemented with isoleucine (Figure 6). Optimal growth was obtained when the expression of either recombinant enzyme was induced by the presence of 50 μM isopropyl 1-thio-β-D-galactopyranoside (IPTG) in the medium. Cells transformed with plasmid pKS21 grew well at both 30 and 42 °C (Figure 6).

Complementation by both the unfused and GST-fused proteins is in agreement with the results of the *in vitro* assay and shows that the *M. tuberculosis* enzyme can fully substitute for the *E. coli* enzyme. Furthermore, misaminoacylation is unlikely because it would be expected to be toxic (Bedouelle et al., 1990; Inokuchi et al., 1984; Vidal-Cros & Bedouelle, 1992).

M. tuberculosis Isoleucyl-tRNA Synthetase Rescues E. coli from Inhibition by Pseudomonic Acid. Pseudomonic acid (PMA) is believed to bind tightly to the same site as isoleucine on eubacterial isoleucyl-tRNA synthetases (Hughes & Mellows, 1978, 1980; Yanagisawa et al., 1994). As with any tight-binding inhibitor, the concentration at which 50% of the enzyme activity is inhibited (IC_{50}) is equal to 50% of the enzyme concentration. Thus, in assays with nanomolar concentrations of *E. coli* enzyme, the IC_{50} is typically in the nanomolar or subnanomolar range. Although *M. tuberculosis* isoleucyl-tRNA synthetase is a eubacterial

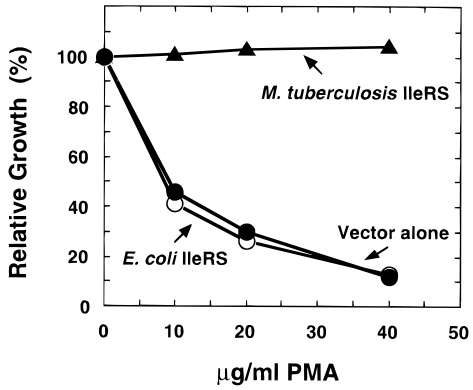


FIGURE 7: Rescue of *E. coli* from pseudomonic acid by *M. tuberculosis* isoleucyl-tRNA synthetase. *E. coli* strain Novablue transformed with the plasmids pBSKS (vector alone), pKS21 (encoding *E. coli* isoleucyl-tRNA synthetase), or pKS56 (encoding *M. tuberculosis* isoleucyl-tRNA synthetase) was grown as described in Materials and Methods, and A_{600} was read after a 36 h incubation at 30 °C. For each strain, percent relative growth was determined as [(A_{600} with PMA)/(A_{600} without PMA)]. In separate experiments, cells expressing *M. tuberculosis* GST−isoleucyl-tRNA synthetase (pGX56) behaved similarly to those containing plasmid pKS56, and *E. coli* strain DH5α gave results similar to those shown for Novablue.

enzyme, we observed no inhibition of its activity with up to 250 μM PMA concentrations (data not shown).

Because PMA inhibits the growth of *E. coli*, and because *M. tuberculosis* isoleucyl-tRNA synthetase functions in *E. coli*, we tested whether the expression of *M. tuberculosis* isoleucyl-tRNA synthetase in *E. coli* would confer resistance to PMA. Growth of DH5α cells expressing wild-type *E. coli* isoleucyl-tRNA synthetase from plasmid pKS21 was completely inhibited when the medium contained 40 μg/mL (80 μM) PMA, while the same cells expressing the *M. tuberculosis* isoleucyl-tRNA synthetase gene encoded by plasmid pKS56 showed no inhibition of growth (Figure 7). In fact, *E. coli* cells harboring plasmid pKS56 continued to grow even in the presence of 640 μg/mL (1.28 mM) PMA (data not shown). Higher concentrations were not tested. Thus, the eukaryotic-like *M. tuberculosis* isoleucyl-tRNA synthetase rescues *E. coli* from the eubacterial-specific drug PMA.

DISCUSSION

The isoleucyl-tRNA synthetase gene of *M. tuberculosis* was characterized and expressed in *E. coli*. The sequence alignments presented in Figure 2A−C show the close relationship of *M. tuberculosis* isoleucyl-tRNA synthetase to cytoplasmic eukaryote rather than to eubacterial or mitochondrial isoleucyl-tRNA synthetases. Because we also found a close relationship between the sequence for *M. tuberculosis* isoleucyl-tRNA synthetase and the sequences obtained here for *M. kansasii* and *M. smegmatis* isoleucyl-tRNA synthetases, we believe that most if not all mycobacterial species may have a eukaryote-like isoleucyl-tRNA synthetase enzyme. No published sequences of other mycobacterial tRNA synthetases are available, but unpublished sequences of leucyl-, methionyl-, and seryl-tRNA synthetases (S. Martinis, unpublished data) and tyrosyl-tRNA synthetase (F. Houman and S. Nair, unpublished data) from *M. tuberculosis* show that these enzymes are closely related to their conterparts in Gram-positive bacteria. Thus, the evolutionary history of mycobacterial isoleucyl-tRNA synthetases may be distinct from that of other tRNA synthetases.

In particular, the eukaryote-like features of *M. tuberculosis* isoleucyl-tRNA synthetase suggest that it may have been acquired by horizontal gene transfer. Evidence for genetic exchange in the evolution and development of tRNA synthetases has been proposed for glutaminyl-tRNA synthetases from Gram-negative eubacterial organisms such as *E. coli* (Lamour et al., 1994). For example, among other considerations, the sequence of *E. coli* glutaminyl-tRNA synthetases is more related to that of mammalian glutamyl- than that of glutaminyl-tRNA synthetase, suggesting that the progenitor in Gram-negative organisms for the glutamine enzyme (which is absent from Gram-positive organisms) was a eukaryotic glutamyl-tRNA synthetase (Nureki et al., 1995). Because mycobacterial organisms such as *M. tuberculosis* grow within a eukaryote host cell, the conditions for gene transfer are favorable. Why the gene for isoleucyl and not for other tRNA synthetases would be acquired in this way is not clear, but an inhibitor (in the host cell) of eubacterial isoleucyl-tRNA synthetase could provide selective pressure for the acquisition of a drug-resistant host cell enzyme. Alternatively, the *M. tuberculosis* gene could have evolved from the same ancestral gene that led to the isoleucyl-tRNA synthetase gene in eukaryotes.

Our observation that *M. tuberculosis* isoleucyl-tRNA synthetase protected *E. coli* from pseudomonic acid is a laboratory demonstration of how horizontal transfer of an essential gene can rescue an organism from a cytotoxic drug. Critical to the success of this experiment was the ability of the pseudomonic acid-resistant *M. tuberculosis* enzyme to cross-acylate *E. coli* $tRNA^{Ile}$ *in vivo*, with apparent high specificity. Similarly, *S. aureus* is sensitive to pseudomonic acid by virtue of potent inhibition of its isoleucine enzyme. *S. aureus* strains that are resistant to high levels of the drug PMA emerged in the wild as a consequence of the acquisition of an episomal gene encoding a novel isoleucyl-tRNA synthetase (Dyke et al., 1991; Rahman et al., 1990) (designated as Sa-2 in Figures 2 and 3).

Our analysis of the various sequences for isoleucyl-tRNA synthetases suggests that the sequence of the extrachromosomal Sa-2 isoleucyl-tRNA synthetase is most closely related to that of the archaebacterial *M. thermoautotrophicum* enzyme (Figures 2 and 3). We infer that this novel episome-encoded synthetase in *S. aureus*, like *M. tuberculosis* isoleucyl-tRNA synthetase in *E. coli*, must acylate the host $tRNA^{Ile}$ with high specificity. Were it not for accurate cross-species acylation, trans-dominant drug resistance could not occur and the selective pressure for acquisition of an exogenous gene for a tRNA synthetase would be eliminated. This consideration suggests an additional or alternative explanation for why the gene for isoleucyl and not for other tRNA synthetases could have been selectively acquired by mycobacterial organisms by a gene transfer mechanism.

This explanation is based on the observation that not all tRNA synthetases exhibit cross-species aminoacylation. For example, mammalian and *E. coli* glycyl-tRNA synthetases do not cross-acylate their respective glycine tRNAs (Shiba et al., 1994a). The reason for the failure to cross-acylate appears to be due to a coadaptation of the sequences of the synthetases to changes in one or more tRNA nucleotides that are essential for aminoacylation. Thus, mammalian $tRNA^{Gly}$ has an A73 "discriminator base" at the end of the acceptor stem, while *E. coli* $tRNA^{Gly}$ has U73 (Steinberg et al., 1993). This nucleotide is essential for aminoacylation. A simple

U73A interchange is sufficient to switch species-specific aminoacylation of microhelix substrates whose sequences are based on the acceptor stem of $tRNA^{Gly}$ (Hipps et al., 1995). Similarly, yeast phenylalanyl-tRNA synthetase does not cross-acylate *E. coli* $tRNA^{Phe}$ unless a critical nucleotide is substituted (Sampson et al., 1989). Thus, the inability of some synthetases to catalyze cross-species aminoacylation may have prevented their genes from being acquired by horizontal transfer.

The structural basis for resistance to high concentrations of pseudomonic acid is not known, but at least some resistance can come from replacement of the highly conserved F594 with leucine (numbering according to the *E. coli* enzyme) in the sequence of type 1 isoleucyl-tRNA synthetases (Yanagisawa et al., 1994). Type 2 sequences, including that of *M. tuberculosis* isoleucyl-tRNA synthetase, replace F594 with I or L (Figure 2B). However, we found that *in vitro* the *M. tuberculosis* isoleucyl-tRNA synthetase was resistant to much higher concentrations of pseudomonic acid (more than 200 μM) than that reported for the *E. coli* mutant F594L isoleucyl-tRNA synthetase (K_i = 17 nM) (Yanagisawa et al., 1994). Thus, more than one of the differences between type 1 and type 2 sequences is likely to be needed to generate resistance to high concentrations of the drug.

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