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Methionyl-tRNA synthetase from *Bacillus stearothermophilus*: structural and functional identities with the *Escherichia coli* enzyme

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ABSTRACT

The *metS* gene encoding homodimeric methionyl-tRNA synthetase from *Bacillus stearothermophilus* has been cloned and a 2880 base pair sequence solved. Comparison of the deduced enzyme protomer sequence (Mr 74,355) with that of the *E.coli* methionyl-tRNA synthetase protomer (Mr 76,124) revealed a relatively low level (32%) of identities, although both enzymes have very similar biochemical properties (Kalogerakos,T., Dessen,P., Fayat,G. and Blanquet,S. (1980) *Biochemistry* 19, 3712 – 3723). However, all the sequence patterns whose functional significance have been probed in the case of the *E.coli* enzyme are found in the thermostable enzyme sequence. In particular, a stretch of 16 amino acids corresponding to the CAU anticodon binding site in the *E.coli* synthetase structure is highly conserved in the *metS* sequence. The *metS* product could be expressed in *E.coli* and purified. It showed structure-function relationships identical to those of the enzyme extracted from *B.stearothermophilus* cells. In particular, the patterns of mild proteolysis were the same. Subtilisin converted the native dimer into a fully active monomeric species (62 kDa), while trypsin digestion yielded an inactive form because of an additional cleavage of the 62 kDa polypeptide into two subfragments capable however of remaining firmly associated. The subtilisin cleavage site was mapped on the enzyme polypeptide, and a gene encoding the active monomer was constructed and expressed in *E.coli*. Finally, trypsin attack was demonstrated to cleave a peptidic bond within the KMSKS sequence common to *E.coli* and *B.stearothermophilus* methionyl-tRNA synthetases. This sequence has been shown, in the case of the *E.coli* enzyme, to have an essential role for the catalysis of methionyl-adenylate formation.

INTRODUCTION

Recent results have led to the partition of aminoacyl-tRNA synthetases into two classes (1, 2). This classification is based in particular on the presence of small stretches of conserved sequences. Class I synthetases, featuring a nucleotide binding fold, have two conserved patterns. The functional relevance of the first pattern, the HIGH sequence (3), has been established in the case of the tyrosyl-tRNA synthetase of *Bacillus stearothermophilus* (4). The second conserved pattern, KMSKS, has been identified by means of affinity labelling using reactive derivatives of tRNA (5, 6) as well as of ATP (7).

The second lysine in KMSKS has been demonstrated by site-directed mutagenesis of the *E.coli* methionyl-tRNA synthetase gene to participate in the stabilization of the transition state during the formation of methionyl adenylate (8). Other regions of functional importance have been identified for this synthetase (9–11). In particular, concerning tRNA recognition, a tryptophanyl residue (Trp461) and the surrounding residues were shown to play a key role in the switching of the binding of tRNAs having either a methionine or an amber anticodon (10).

In order to probe the functional relevance of these amino acid patterns, it was of interest to determine whether or not they occurred in the sequences of methionyl-tRNA synthetases of other origins. For this purpose, we cloned and sequenced the gene of *B.stearothermophilus* encoding methionyl-tRNA synthetase, because this enzyme possesses biochemical and functional properties closely resembling those of the *E.coli* enzyme. This approach was also expected to highlight other conserved patterns of potential significance.

Methionyl-tRNA synthetases from *E.coli* (MTS) as well as from *B.stearothermophilus* (MTSBS) are homodimeric enzymes. Each of these dimeric synthetases can be converted into a fully active monomeric fragment of reduced Mr by mild proteolysis (12, 13). In the case of the *E.coli* enzyme, this conversion can be obtained with a variety of proteases, including trypsin and subtilisin. The fragment generated by trypsic digestion (64 kDa)

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has been crystallized and a tridimensional model at 2.5 Å resolution is now available (14). A similar conversion of the thermostable enzyme into a fragment of 62 kDa could be obtained with subtilisin, whereas digestion by trypsin yielded an inactive form (13). This loss of activity was due to an additional cleavage of the 62 kDa polypeptide into two subfragments of 33 kDa and 29 kDa. capable however of remaining firmly associated under non-denaturing conditions.

The sequence of the *B.stearothermophilus* enzyme reported here displays a small level of identities with the *E.coli* enzyme. However, all the patterns the functional significances of which have been established in the case of the *E.coli* enzyme are present in the sequence of the thermostable enzyme.

MATERIALS AND METHODS

Strains and general techniques

E.coli strains JM101Tr (15), a *recA* derivative of JM101 (16), and XLI-Blue (Stratagene) were used to host the various plasmids and M13 phages studied. General genetic and molecular biology techniques were as described (17, 18). When required, DNA restriction fragments were purified using high performance liquid chromatography (19).

Nucleotides, DNA restriction and modification enzymes were purchased from Boehringer (Mannheim) or Pharmacia, France. Oligonucleotides were synthesized by the β-cyanoethyl phosphoramidite method with a Gene-Assembler (Pharmacia), and purified by ion-exchange chromatography (MonoQ, Pharmacia). Site directed mutagenesis was performed as previously described (20).

The mixed probe representing any possible coding sequence corresponding to the N-terminus of MTSBS was 5'ATGG-A(A,G)AA(A,G)AA(A,G)AC(A,G,C,T)TT(T,C)TA3'. The probe used to clone the 3'-end of the methionyl-tRNA synthetase gene was 5'GACTTGGCTGTATCGCGGACGACGTTTGAC-TGGGGCAT3'. The mutagenic oligonucleotide used to introduce a *KpnI* site in front of the *metS* gene encoding the *B.stearothermophilus* methionyl−tRNA synthetase was 5'TGCGCACAAAGGTACCTGTCCTGA3'. The mutagenic oligonucleotide used to introduce an opal codon at position 535 in the *metS* coding sequence was 5'GCGGCCAAATGAGAA-AAACAA3'. DNA sequencing of the *metS* region was from restriction fragments cloned in M13mp phages (16) using universal or internal oligonucleotidic primers.

Methionyl-tRNA synthetase activity measurements

To measure the specific activity of methionyl-tRNA synthetase in *E.coli* crude extracts, overnight cultures of JM101Tr cells carrying the desired plasmid were harvested by centrifugation, and suspended in 20 mM Tris-HCl (pH 7.6), 0.1 mM dithiotreitol, 0.1 mM EDTA. Cells were disrupted by sonication. tRNA aminoacylation and/or [^{32}P]PPi-ATP isotopic exchange activities were assayed as described (21). In some cases, activities were assayed after a 20 min. incubation of the extract at 65°C, which insured inactivation of the endogenous *E.coli* methionyl-tRNA synthetase.

Preparation of *Bacillus stearothermophilus* chromosomal DNA

B.stearothermophilus strain ATCC1518 was grown at 60°C in LB medium supplemented with 0.25% casaminoacids (Difco), 0.5 mM calcium chloride, 0.05 mM manganese chloride, 4 μM ferric chloride, 0.8 mM magnesium sulfate, 1% glucose and buffered at pH 7.3 with 75 mM potassium phosphate (22). Cells from a one litre culture were harvested at 1 OD_{650} and washed with 1 litre of 15 mM sodium citrate buffer (pH 7) containing 150 mM sodium chloride. Cells were resuspended in 23 ml of 50 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, 20% sucrose and 1% lysozyme, and incubated 15 minutes at room temperature (23). Lysis was achieved by adding 40 ml of 0.125 M EDTA, 1% lauryl sarcosylate (pH 9.6), and pronase (10 mg/ml final concentration). After incubation at 50°C, 40 ml of phenol/chloroform (1:1 v/v) were added, and the extracted aqueous phase was then treated with ether. Chromosomal DNA was finally purified by isopycnic centrifugation through a gradient of caesium chloride.

To perform hybridization experiments, high molecular weight *B.stearothermophilus* chromosomal DNA (30 μg) was digested with restriction enzymes, submitted to electrophoresis on a 0.8% agarose gel, and transferred onto nitrocellulose filters (Schleicher and Schüll — BA85). Hybridization was performed under non-stringent conditions, and the filters were washed at increasing temperatures in 6×SSC buffer.

Purification of *B.stearothermophilus* methionyl-tRNA synthetase produced in *E.coli* cells

JM101Tr cells carrying pMBSIII (1 litre) were grown overnight at 37°C in LB containing 0.1 mg/ml ampicillin and 1 mM IPTG. Cells were harvested, resuspended in 120 ml of 10 mM potassium phosphate (pH 7.3) and sonicated. After centrifugation, sodium ethylenediamine tetraacetate (EDTA, 5 mM final concentration) and streptomycin sulphate (0.5% w/v) were added to the supernatant. After centrifugation, the supernatant was incubated at 65°C for 20 min and the soluble proteins were fractionated by $(NH_4)_2SO_4$ precipitation (45 to 60% saturation). The protein solution was applied onto a Sephacryl S-300 (Pharmacia) column (1 cm×120 cm) equilibrated in buffer A (10 mM potassium phosphate pH 7.3; 10 mM 2-mercaptoethanol) and eluted at 0.2 ml/min. Pooled active fractions were applied onto a 5 ml DEAE Spherodex M column (IBF, France) equilibrated in buffer A. After that, a 0 to 1 M KCl gradient (2 ml/min, 0. 17 mM/min) was performed. The enzyme was finally purified onto a Superose 6 column (Pharmacia, 1.6 cm×50 cm) equilibrated in buffer A plus 50 mM KCl.

Mild proteolysis of methionyl-tRNA synthetase

MTSBS (1 mg/ml) was incubated at 37°C in 0.1 M Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol, in the presence of subtilisin (Sigma, 1/500 w/w) or trypsin (Sigma, 1/1000 w/w). After a 1 hour incubation, the proteolysis was quenched by adding phenylmethylsulfonyl fluoride (0.1 mM final concentration) in the case of subtilisin digestion, or egg-white ovomucoid (2:1 w/w with respect to trypsin) in the case of trypsinolysis. The reaction products (100 to 300 ng; 1.3 to 4 pmoles) were analyzed by SDS−polyacrylamide gel electrophoresis (12.5% homogeneous gels, Phast-System, Pharmacia).

Isolation of peptides

MTSBS proteolytic digests (5 nanomoles) were applied to an Aquapore RP300 column (100 mm×2.1 mm) equilibrated in eluent B and separated with a 40 min. linear gradient from 0 to 80% eluent C (eluent B: 0.1% trifluoroacetic acid; eluent C: 20% isopropanol and 60% acetonitrile in 0.1% trifluoroacetic acid); the flow rate was 0.25 ml/min. Peptides were detected through measurements of UV absorbance (190−370 nm) with

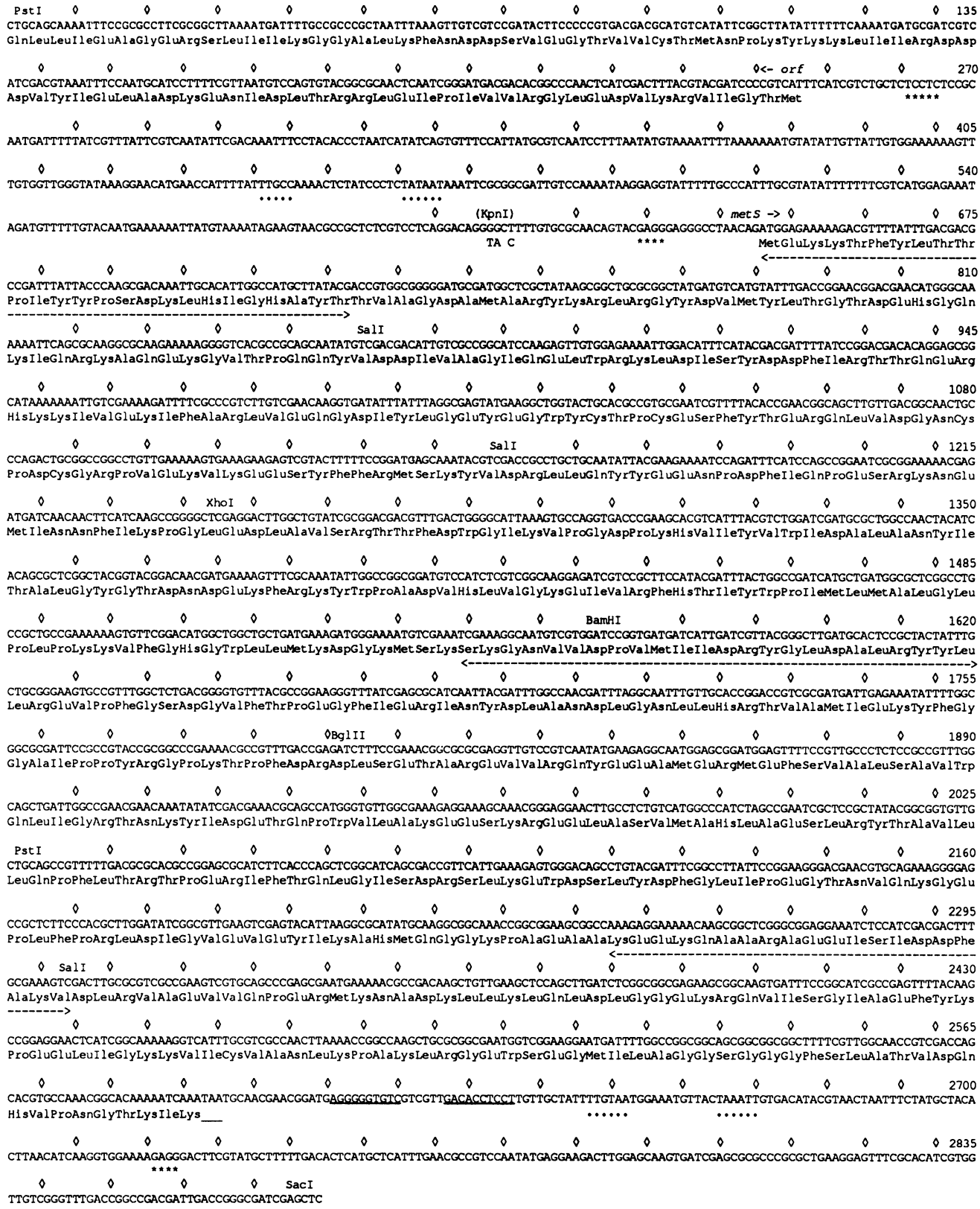


Figure 1. DNA sequence of *metS* and of the surrounding regions. The 2880 bp DNA sequence between the *PstI* and *SacI* cloning sites was determined on both strands by the dideoxy chain termination technique. The relevant restriction sites are indicated above the sequence. The nucleotides substituted to create a *KpnI* site are illustrated below the wild-type sequence (region 610). The deduced amino acid sequence of methionyl-tRNA synthetase is indicated by '*metS*'. The upstream open reading frame on the opposite DNA strand encoding a peptide homologous to the *B.subtilis abrB* gene product is also translated. The amino acid sequences determined by chemical degradation are indicated by the dotted arrows below the translated *metS* DNA sequence. The putative ribosomal binding sites are depicted by asterisks. Sequences homologous to the prokaryotic promoter consensus are indicated by dots. The potential transcriptional terminator of *metS* is underlined.

a diode array detector (Applied Biosystems Model 1000S) and fluorescence emission at 330 nm (excitation wavelength 290 nm).

N-terminal sequencing

Automated Edman degradation was carried out on a gas-phase sequencer (Applied Biosystems Model 470A). Protein and peptide samples (30 µl, 0.3–1.0 nmol) were loaded on glass fibre filters treated with 2.5 mg of Biobrene (Applied Biosystems). Phenylthiohydantoin amino acids were identified as already described (24).

RESULTS

Molecular cloning and sequencing of the methionyl-tRNA synthetase gene from *Bacillus stearothermophilus*

In order to design mixed oligonucleotide probes, the N-terminal amino acid sequence of the methionyl-tRNA synthetase (MTSBS) previously purified from the *Bacillus stearothermophilus* strain ATCC1518 (13) was determined by automated Edman degradation. According to the analysis of the sequence of the 26 first amino acids (MEKKTFYLTTPIYYPSDKLHIGHAYT; Figure 1), a mixture of 64 twenty-mer oligonucleotides representing all the coding sequences corresponding to the N-terminus MEKKTFY peptide was prepared.

High molecular weight *B.stearothermophilus* chromosomal DNA was digested with either *Eco*RI, *Hin*dIII, or *Pst*I, and submitted to electrophoresis prior to Southern hybridization using the [^{32}P]-labelled degenerated DNA probe. A single *Pst*I band of 2 kb retaining radioactivity after washing at 37°C was reproducibly observed. High performance size exclusion chromatography of *Pst*I restricted chromosomal DNA (100 µg) produced a fraction enriched in the identified *Pst*I fragment (19). 25 ng of this preparation was ligated with 100 ng of *Pst*I-linearized Bluescript M13+KS DNA. After transformation of XLI-Blue cells, 2000 clones were obtained. Plasmidic DNA from 100 randomly chosen clones was prepared and restricted by *Pst*I. The restricted DNA was analyzed by electrophoresis, and submitted to Southern hybridization analysis using the oligonucleotidic probe. One positive plasmid, having the structure of pMBSI (Figure 2), was found among the first 70 recombinant plasmids analyzed. In order to verify that the cloned 2 kb fragment was the same as that identified in the genomic blotting experiment, a labelled single stranded DNA probe was prepared

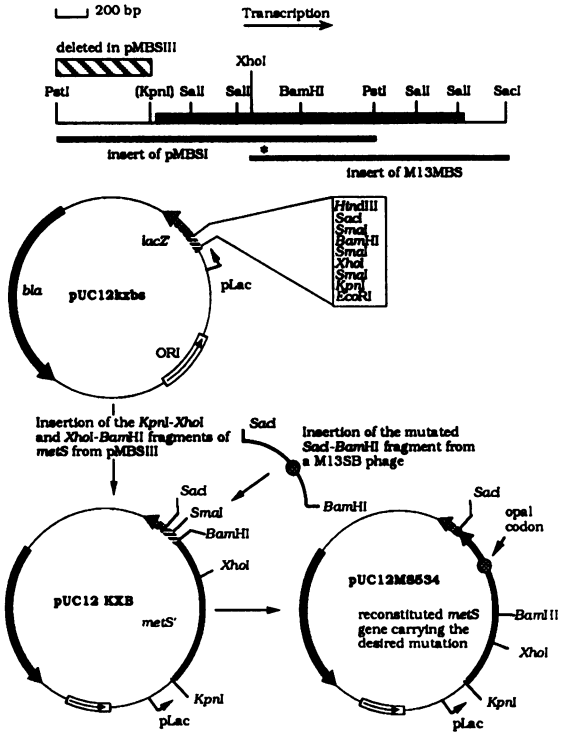


Figure 2. Vectors used to express MTSBS in *E.coli*. The upper part of the figure represents the *metS* region reconstituted between the *Pst*I and *Sac*I sites in a Bluescript M13+KS vector to give the recombinant plasmid pMBSII. The grey box symbolizes the *metS* gene. The *Pst*I and *Xho*I–*Sac*I fragments initially cloned in Bluescript or M13mp18 vectors are represented by solid horizontal lines. The asterisk shows the position of the oligonucleotide probe used to isolate M13MBS. The *Kpn*I site created by oligonucleotide site-directed mutagenesis using pMBSII as a matrix is indicated in parentheses. The hatched box depicts the *Kpn*I–*Kpn*I DNA fragment subsequently excised to generate plasmid pMBSIII. The lower part of the figure schematizes the strategy used to construct a *metS* opal mutant. For this purpose, a specialized vector, pUC12kxbs, was constructed by replacing the pUC12 polylinker by the depicted one. From this vector, three *metS* plasmids were derived, each lacking one of the following regions: *Kpn*I–*Xho*I, *Xho*I–*Bam*HI, or *Bam*HI–*Sac*I. The pUC12KXB plasmid lacking the *Bam*HI–*Sac*I fragment was used in this study. This latter fragment, cloned in a M13 phage. was submitted to oligonucleotide site-directed mutagenesis before its insertion between the corresponding sites of pUC12KXB to give pUC12MS534 carrying a *metS* gene with the desired opal codon at position 535.

	- IPTG	+ IPTG
Bluescript	0 / 2.6 (a)	0 / 2.2 (a)
pMBSI	0	0
pMBSII	55	59
pMBSIII	12	40 / 41 (a)

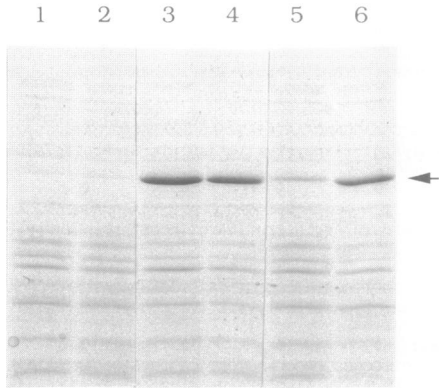


Figure 3. Expression of native dimeric MTSBS in *E.coli*. Upper part: the specific activities of tRNA methionylation measured from crude extracts of JM101Tr cells carrying the indicated plasmids are shown. Cells were grown overnight at 37°C in LB medium containing 50 µg/ml of ampicillin, in the presence (+ IPTG) or in the absence (− IPTG) of 1 mm IPTG. The extracts were heated 20 minutes at 65°C prior to measuring the aminoacylation activity. Rate values are expressed as picomoles of $tRNA^{Met}$ aminoacylated per second per ml of extract having an absorbance of 1 at 280 nm. (a) rate values obtained without prior heating of the extracts at 65°C. Lower part: the PAGE analysis under denaturing conditions of the above extracts are shown in the lower part of the figure. Shown are: crude extracts of cells carrying pMBSI grown in the absence (lane 1) or in the presence (lane 2) of IPTG; crude extracts of cells carrying pMBSII grown in the absence (lane 3) or in the presence (lane 4) of IPTG; crude extracts of cells carrying pMBSIII grown in the absence (lane 5) or in the presence (lane 6) of IPTG. The arrow on the right indicates the migration of pure *B.stearothermophilus* methionyl-tRNA synthetase. The direction of migration is from the top to the bottom of the figure.

using the cloned 2 kb fragment as a matrix. The hybridization patterns of this probe and of the oligonucleotidic probe with *B.stearothermophilus* DNA blots were identical. This confirmed that the cloned fragment was indeed the one previously identified on the genomic blot.

The DNA sequence of the *Pst*I fragment was established. It revealed an open reading frame encoding 462 residues, the 5'-end of which specified an amino acid sequence identical to the microsequenced N-terminus of the MTSBS. However, this open reading frame did not end within the cloned fragment, demonstrating that only a fraction of the desired gene was present (Figures 1 and 2). Accordingly, no thermostable methionyl-tRNA synthetase activity could be detected in crude extracts prepared from *E.coli* cells harbouring the pMBSI plasmid (Figure 3).

To clone the 3'-end of the *metS* gene, a 38-mer oligonucleotide was synthesized on the basis of a sequence located on the 3'-side of a *Xho*I site shown in Figure 2. This oligonucleotide was used as a hybridization probe to identify a 1.7 kbp *Xho*I-*Sac*I fragment on genomic blots. Using the same strategy as above, a recombinant M13mp18 phage, M13MBS, was isolated by *in situ* hybridization on phage plaques. DNA sequencing established that the cloned fragment indeed contained the 3'-end of the *metS* gene (Figure 1). Reconstruction of the whole gene was achieved by inserting the 1571 bp *Pst*I-*Bam*HI fragment from pMBSI together with the 1308 bp *Bam*HI-*Sac*I fragment from M13MBS, between the *Pst*I and *Sac*I sites of Bluescript M13$^+$KS. The resulting plasmid, carrying a 1947 base coding sequence, was called pMBSII (Figure 2).

The *metS* sequence and flanking regions are shown in Figure 1. The *metS* structural gene is composed of 649 codons and accounts for a polypeptide of Mr 74,355. In front of *metS* is a GAGG sequence indicative of a ribosomal binding site. This region is preceded 180 bases upstream by a sequence homologous to the prokaryotic promoter consensus (bases 441−446 and 461−466).

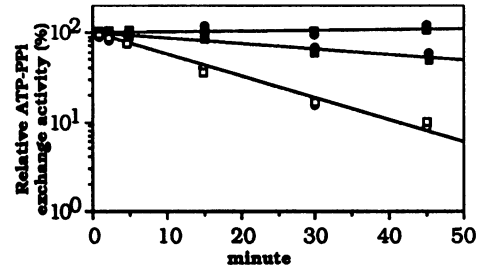
In the case of the *E.coli* methionyl-tRNA synthetase gene (*met*G), the leader RNA region contains a tRNA^{Met}-like structure followed by a transcription terminator (25). These secondary structures are believed to be involved in the control of the expression of *metG*. We examined the 650 bp region upstream from the *metS* initiator codon, but were unable to recognize any stable secondary structure reminiscent of those observed with *metG*. Four hundred bp upstream from the start codon of *metS*, an open reading frame is found on the complementary DNA strand. It comprises 82 codons, and does not end within the cloned fragment. Comparison of the corresponding amino acid sequence with those available in the GENBANK data library (release 66) revealed 85% of identities with the N-part of the product of the *B.subtilis abrB* gene (26), a 96 residue DNA binding protein involved in the transcriptional repression of stationary phase-induced genes (27, 28). It is therefore tempting to propose that this open reading frame corresponds to the 5'-end of a *B.stearothermophilus* gene encoding a protein with a function equivalent to that of the *B.subtilis abrB* product. Moreover, 500 bp upstream from the *abrB* gene of *B.subtilis*, the 5'-end of a divergent open reading frame showing very strong similarities to the amino terminal portion of *E.coli* methionyl-tRNA synthetase was mentioned (26). Altogether, these observations indicate similar genomic organizations of the *metS* regions in both *B.subtilis* and *B.stearothermophilus*.

The RNA region following the *metS* coding sequence can be folded into a stem and loop structure, resembling a rho-independent transcription terminator (bases 2610−2650 in Figure 1). This region is followed by an open reading frame (start codon at bases 2733−2735) which does not end within the cloned fragment. This open reading frame is preceded by a possible ribosomal binding site (bases 2721−2725) as well as by a sequence homologous to a prokaryotic promoter consensus (bases 2668−2710).

Expression of the *B.stearothermophilus metS* gene in *E.coli*

JM101Tr cells transformed by the pMBSII plasmid overproduced the activity of tRNA methionylation by a factor of twenty when compared to cells harbouring the Bluescript M13$^+$KS vector. This overproduced activity remained intact upon a 20 minute heating of the crude extract at 65°C prior to enzyme assay, whereas, in the control extract, the tRNA methionylation activity vanished (Figure 3).

Although the *metS* transcription in pMBSII was potentially under the control of the inducible Lac promoter, addition of 1 mM IPTG to the culture medium had no significant effect on enzyme overproduction (Figure 3). As an attempt to render inducible the *metS* expression, the 649 bp leader region was deleted. For this purpose a *Kpn*I site was created 25 bp upstream from the ATG codon of *metS* by site-directed mutagenesis, and advantage was taken of the *Kpn*I site of the Bluescript polylinker to excise the 605 bp leader region. The resulting plasmid, pMBSIII (Figure 2) was introduced into JM101Tr cells. As shown by activity measurements and PAGE analysis (Figure 3) the *metS*



	half lifetime (minutes) at			
	51°C	60°C	70°C	75°C
MTSBS produced in *B. stearo.*	n.d.	> 400	50	14
MTSBS produced in *E. coli*	n.d.	> 400	50	14
MS534 produced in *E. coli*	n.d.	> 400	> 400	> 400
MTSn from *E. coli*	9	n.d.	n.d.	n.d.
M547 from *E. coli*	> 400	1	n.d.	n.d.

Figure 4. Comparison of the thermal stabilities of MTSBS produced from either *E.coli* or *B.stearothermophilus*. Panel A: native dimeric MTSBS (120 nM) produced from *E.coli* (circles) or *B.stearothermophilus* (squares) was incubated in 20 mM potassium phosphate buffer (pH 7.0) at 60°C (filled symbols), 70°C (grey symbols) or 75°C (open symbols). At various times, aliquots were withdrawn and assayed for [^{32}P]PPi-ATP isotopic exchange activity at 25°C. Residual initial velocities (%) are plotted in logarithm scale *versus* the time of incubation at high temperature. Panel B: shown are the half lifetimes of [^{32}P]PPi-ATP isotopic exchange activity at the indicated temperatures of various *E.coli* and *B.stearothermophilus* MTS, as deduced from experiments similar to those shown in panel A. MS534 is an active truncated monomeric form of MTSBS expressed in *E.coli* from a *metS* derivative composed of the 534 first codons. MTSn is the native dimeric enzyme from *E.coli* and M547 is its active truncated monomeric form composed of 547 residues (21). n.d.: not determined.

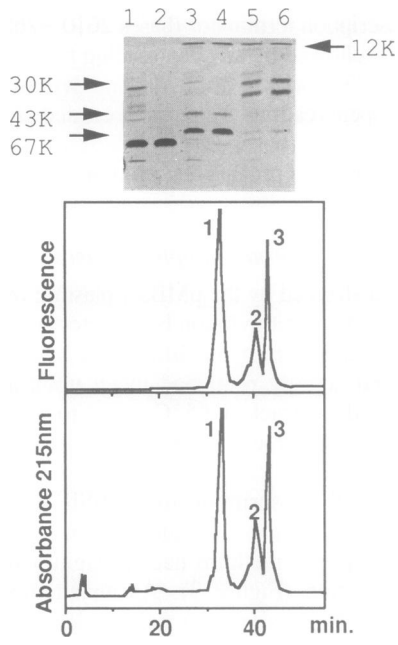


Figure 5. Mild proteolysis of native MTSBS produced from *E.coli* or *B.stearothermophilus*. Upper part: Native MTSBS produced from *B.stearothermophilus* or *E.coli* was submitted to mild proteolysis with either subtilisin or trypsin, as described in 'Materials and Methods'. The reaction products (0.5 μg) were analyzed by SDS−PAGE (12.5% of acrylamide, Phast-System, Pharmacia). Lane 1: native MTSBS purified from *B.stearothermophilus*; lane 2: native MTSBS produced from *E.coli*; lanes 3 and 4: subtilisin digestion of native MTSBS produced from *B.stearothermophilus* and *E.coli*, respectively; lanes 5 and 6: trypsin digestion of native MTSBS expressed from *B.stearothermophilus* or *E.coli* respectively. The migrations of three molecular weight markers are indicated by arrows on the left. The arrow on the right indicates the 12 Kd peptides, the Mr of which was determined by using a 20% acrylamide gel (data not shown). Lower part: The purified 62 kDa subtilisic fragment of MTSBS was submitted to mild trypsin digestion. The reaction products were separated on an Aquapore RP300 column, with a 40 min linear gradient from, 0 to 80% eluent B (eluent A: 0.1% trifluoroacetic acid; eluent B: 20% isopropanol, 60% acetonitrile in 0.1% trifluoroacetic acid); the flow rate was 0.25 ml/min. The UV absorbance (215 nm) and fluorescence (emission at 330 nm; excitation wavelength 290 nm) profiles are shown. The peptides corresponding to peaks 1, 2 and 3 were identified by SDS−PAGE analysis as: undigested 62 kDa fragment (peak 2), 33 kDa fragment (peak 1) and 29 kDa fragment (peak 3).

expression from this plasmid became inducible by the addition of IPTG.

To establish that the MTSBS produced in *E.coli* was identical to that isolated from the *B.stearothermophilus* cells, purification of the MTSBS overproduced in *E.coli* was undertaken. Following the procedure described in Materials and Methods, 13 mg of homogeneous MTS were obtained from a one litre culture of strain JM101Tr-pMBSIII. As shown in Figure 4, MTSBS from *E.coli* origin exhibited the same stability against thermal denaturation as the enzyme expressed in the thermophilic bacillus. Moreover, the rates of the ATP-PPi isotopic exchange (4 s^{-1}) and tRNA aminoacylation (0.8 s^{-1}) reactions were identical. These data rendered unlikely the possibility that the thermal stability of the *metS* product could have resulted from post-translational modifications specific to *B.stearothermophilus*.

Mapping of the sites hypersensitive to proteolytic attack

Mild proteolysis of the *E.coli* dimeric enzyme with either trypsin or subtilisin converts it into an active monomeric fragment of 62.3 kDa (547 N-terminal residues). In contrast, the thermostable enzyme can be cleaved into a 62 kDa active monomer only with subtilisin, whereas digestion by trypsin yields an inactive species.

Mild subtilisin and trypsin digestions of the MTSBS expressed in *E.coli* yielded a polypeptide pattern indistinguishable from that previously obtained with MTSBS isolated from *B.stearothermophilus* (Figure 5). Subtilisin digestion of MTSBS from either origin resulted in the accumulation of two major bands (62 kDa and 12 kDa; Figure 5, lanes 3 and 4). The 62 kDa polypeptide retained full activity. In the case of trypsin digestion, only traces of the 62 kDa polypeptide were observed, while three peptides accumulated: one corresponding to the above 12 kDa species, and two additional peptides of 33 kDa and 29 kDa (Figure 5, lanes 5 and 6). As described earlier, trypsin cleavage caused a complete loss of all enzyme activities, due to the splitting of the 62 kDa polypeptide into two subfragments (33 and 29 kDa), which remain firmly associated under non-denaturating conditions (13).

Mapping of the site of subtilisin cleavage

Native dimeric MTSBS was incubated in the presence of subtilisin as described in Materials and Methods. The two major polypeptides resulting from mild digestion (12 kDa and 62 kDa; Figure 5) were separated by reversed phase liquid chromatography and their N-terminal sequences were determined. The 62 kDa polypeptide proved to have the same N-terminal sequence as the native enzyme. The 12 kDa species eluted as a single peak under the HPLC separation conditions used. However, upon Edman degradation, it turned out to be a mixture of two polypeptides resulting from cleavage of either bond 533−534 or bond 543−544 of the MTSBS native polypeptide. As shown in Figure 6, the two peptide bonds cleaved by subtilisin belong to two highly conserved sequences: ^{534}KEEKQAAR-A^{542}, aligned with the *E.coli* enzyme ^{547}KEEVKAAAA^{555} peptide, and ^{543}EEISIDDF^{550} aligned with the *E.coli* enzyme ^{568}ETITFDDF^{575} sequence. According to these cleavage sites, a C-terminal fragment of 106−116 residues (12 kDa) and a fragment composed of the 533−542 N-terminal residues (61.7 to 62.7 kDa) can be calculated. The Mr values of these fragments are in good agreement with the Mr values deduced from SDS−PAGE analysis (Figure 5).

It should be noted that upon our proteolysis conditions, three additional minor bands (41 kDa, 33 kDa and 29 kDa) were also observed. These products may reflect an additional minor site of cleavage by subtilisin, occurring 33 kDa downstream from the N-end of the enzyme. As will be shown further below, a cleavage site can be revealed at this location when the enzyme is exposed to the action of trypsin instead of subtilisin.

Mapping of the sites of trypsin cleavage

As previously shown (13), the trypsinolysis of the active subtilisic fragment of 62 kDa generates two fragments of 33 kDa and 29 kDa which can be separated only under denaturing conditions. The N-terminal sequences of the subtilisic fragment were analyzed before and after trypsinolysis and showed that trypsin digestion released only one additional N-terminus in a 1:1 stoichiometry with that of the subtilisic fragment. This demonstrated that inactivation of the subtilisic enzyme by trypsin resulted from a single peptidic bond cleavage. This conclusion was reinforced by N-terminal sequencing of each of the subfragments separated by reversed phase liquid chromatography (Figure 5). The 33 kDa fragment exhibited the same amino-terminal sequence as that of the subtilisic or native MTSBS. The

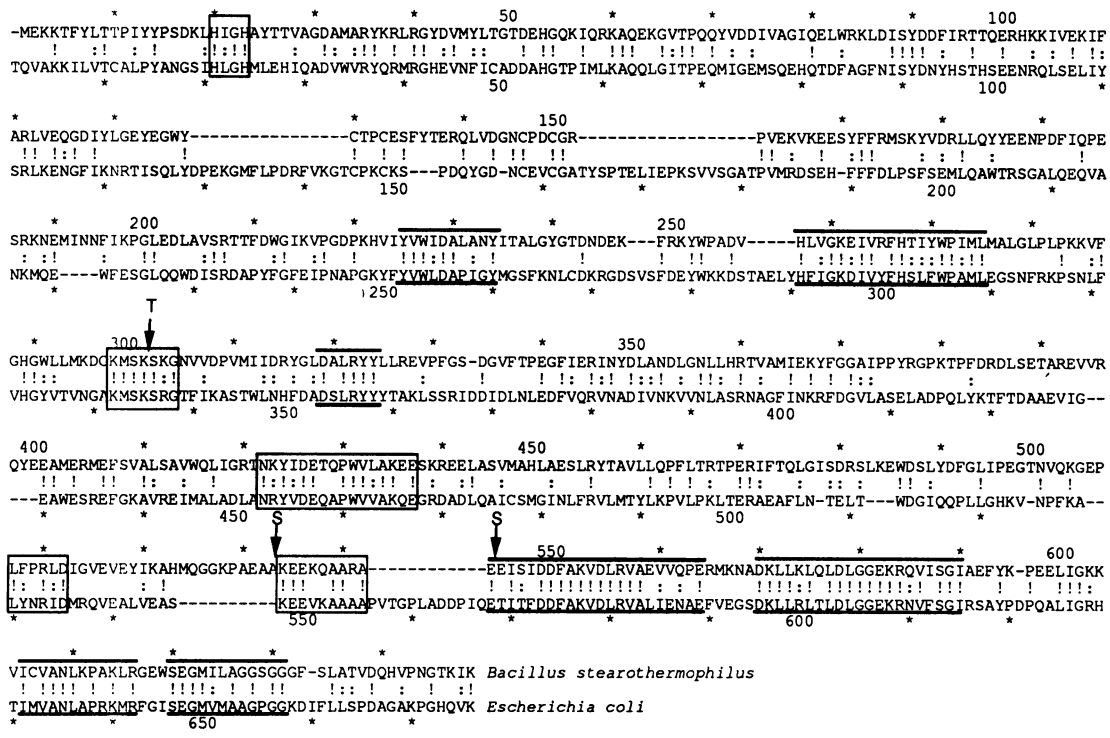


Figure 6. Alignment of the *E.coli* (down) and *B.stearothermophilus* (up) methionyl-tRNA synthetase amino acid sequences. The sequences were processed manually, primarily by aligning the patterns (boxed on the figure), which are of known functional significance. The regions with a high level of identities are indicated by solid horizontal bars. Concerning the regions with a low degree of identity, the chosen alignment maximized the matches of bidimensional patterns, as deduced from hydrophobic cluster analysis (36). The sites of subtilisin (or trypsin) cleavage of MTSBS are indicated by S (or T). Identities are symbolized by an exclamation mark (!), whereas the conservative replacements are signalled by a colon (:).

Ser-Lys-Gly-... N-terminal sequence displayed by the 29 kDa polypeptide indicated that this fragment starts at position 301 (Figure 6). This stretch could be aligned with the ^{336}Ser-Arg-Gly-... sequence of the *E.coli* enzyme (Figure 6). Noticeably, the ^{301}Ser-Arg-Gly^{303} sequence of MTSBS and the ^{336}Ser-Arg-Gly^{338} sequence of *E.coli* MTS overlap a KMSKS pattern, indicative of the catalytic site of class I aminoacyl-tRNA synthetases (8, 29, 30).

Expression in *E.coli* of the active monomeric fragment of MTSBS

A stable active monomeric form of the *E.coli* enzyme (named M547; in (21)) could be produced from a gene having a stop codon after Lys 547 (^{547}KEEVK^{551} sequence). This observation, together with the above results prompted us to introduce an opal stop codon within the conserved ^{534}KEEK^{537} sequence of the MTSBS downstream from the codon for Lys 534. The oligonucleotide site-directed mutagenesis was achieved using a M13 phage carrying the 1312 bp *Bam*HI-*Sac*I fragment. Reconstruction of the *metS* gene was achieved by inserting this mutated fragment between the same sites of a pUC vector carrying the 5'-part of the *metS* gene (Figure 2). *E.coli* cells transformed by the resulting pMS534 plasmid overproduced a 62 kDa active polypeptide (MS534). The functional identity of this species with the subtilisic MTSBS was confirmed after purification to homogeneity. Indeed, the values of the initial rates of the ATP-PPi exchange (5 s^{-1}) and tRNA aminoacylation (0.5 s^{-1}) reactions catalysed by the MS534 enzyme were identical to those (respectively 4.6 and 0.54 s^{-1}) measured with the subtilisic form of MTSBS (13). Finally, the thermal stability of the MS534 enzyme was compared to those of the *E.coli* M547 and native MTSBS forms. Interestingly, in a manner analogous to the *E.coli* methionyl-tRNA synthetase system, the truncated MS534 form exhibited a markedly improved resistance to thermal denaturation, as compared to its native dimeric counterpart. Indeed, half lifetimes of 15 min at 75°C for native MTSBS and >300 min for MS534 were measured, which compare well with half lifetimes obtained at 51°C with *E.coli* enzymes: 16 min for native MTS and >300 min for M547 (12, 21) (Figure 4).

DISCUSSION

Methionyl-tRNA synthetases from *E.coli* and *B.stearothermophilus* were previously shown to share several properties (13). Both native enzymes are homodimers, displaying two equivalent sites for methionine activation. The mechanisms of the methionine activation reaction catalysed by each of the enzymes are very similar. In particular, it was demonstrated that formation of a reactive enzyme:methionine:ATP-Mg^{2+} complex was conditioned by synergistic couplings between the methionine and ATP sites. Finally, the two dimeric enzymes are both capable of binding two tRNA molecules in an anti-cooperative manner.

In the present work, the comparison of the fragments released by mild proteolysis strongly indicates that MTS and MTSBS have very similar structural organizations. In the case of each synthetase, any proteolysis removes a C-terminal domain (109 to 126 residues), the presence of which is required for protomer association. However, while the resulting *E.coli* truncated monomer is resistant to the presence of trypsin, the

B.stearothermophilus enzyme is further cleaved by this protease within the highly conserved KMSKS sequence.

Alignment of the *E.coli* and *B.stearothermophilus* methionyl-tRNA synthetase sequences reveals 32% identical residues. The C-terminal region is the most conserved, with 62 identities out of 120 compared residues. These C-terminal residues can be removed by mild proteolysis without affecting enzyme activity. In the case of *E.coli* as well as of *B.stearothermophilus*, the removal of this C-end markedly increases enzyme thermal stability.

When considering the amino acid sequences of the truncated active monomers, the percentage of identical residues drops to 27%. However, there are islands of identity clustered in several sites. Some of these sites contain patterns which are crucial for the activity of the *E.coli* enzyme.

The HIGH sequence, conserved among class I aminoacyl-tRNA synthetase species, prokaryotic and eukaryotic, cytoplasmic and mitochondrial (1, 2, 3, 31, 32 and references therein), is found at positions 20−23 in the sequence of MTSBS. The functional relevance of this conserved sequence has been established in the case of tyrosyl-tRNA synthetase of *B.stearothermophilus* by using site-directed mutagenesis. The first His residue (His 45) was shown to participate, through interaction with the γ-phosphoryl of ATP, in the stabilization of the transient complex during tyrosyl-adenylate formation. The second His (His 48) is believed to be involved in the binding of the ribose of ATP (4 and references therein).

The ^{298}KMSKS^{302} region of MTSBS, is fully conserved in the sequences of MTS from *E.coli* and *S.cerevisiae* (cytoplasmic and mitochondrial). Like the HIGH sequence, KMSKS is considered as a signature sequence among class I aminoacyl-tRNA synthetases. The present study reveals that the inactivation of the *B.stearothermophilus* methionyl-tRNA synthetase upon trypsin treatment was caused by a cleavage at the level of this KMSKS sequence. A site of trypsin cleavage leading to the inactivation of the truncated monomer was also identified in *T.thermophilus* MTS (30). In this case, trypsin released a 23 kDa fragment with a N-terminal sequence Thr-Leu-Gly-Asn-Val-, similar to the sequence ^{302}Ser-Lys-Gly-Asn-Val-, adjacent to the site of trypsin cleavage of the *B.stearothermophilus* synthetase.

Interestingly, after trypsin digestion, the two fragments created at the expense of the 62 kDa form of MTSBS remain firmly associated. However, the ability to catalyse the formation of the enzyme:methionyl-adenylate complex has disappeared (13). This behaviour caused by a cleavage at the level of KMSKS parallels that of *E.coli* enzyme mutants in which Lys335 (the second Lys in KMSKS) was substituted by a Gln, an Ala, an Arg or a Glu. From the study of these mutants, it was concluded that Lys335 played a key role in the stabilization of the transient complex during formation of the methionyl-adenylate, by interacting with the pyrophosphate moiety of ATP (8). The KMSKS region of the thermostable enzymes seems therefore to have an equivalent function. In addition, the lack of dissociation of the fragments generated by trypsin suggests that they may retain the capacity of making specific contacts in spite of the cleavage. This possibility is reinforced by the observation that the two associated fragments can still bind tRNA, ATP-Mg^{2+} and methionine (13). Such an idea is further supported by the location of the KMSKS motif at a junction between the two domains evidenced in the crystalline structure of the *E.coli* enzyme (14) as well as by the recent demonstration that $tRNA^{Met}$ aminoacylation activity could be recovered after the assembly of two *E.coli* MTS fragments produced from a *metG* gene split at the inter-domain junction (34). This further argues for the structural identity between the two *E.coli* and *B.stearothermophilus* enzymes.

At position 422 in the MTSBS sequence, a stretch of 16 amino acids is found highly conserved in the *E.coli* MTS sequence (Figure 6). A homologous sequence is also present in the yeast mitochondrial enzyme (residues 477−492) (32). In the *E.coli* enzyme, this region and, in particular, the Trp461 residue are thought to be involved in the discrimination of the tRNA anticodon sequence, a key step in the tRNA binding process (10) and its subsequent aminoacylation (9−11). Interestingly, the MTSBS possesses a Trp at position 431, the surrounding of which appears similar to that of the above Trp461 in the *E.coli* sequence. In this context, it is noteworthy that the *B.stearothermophilus* enzyme efficiently aminoacylates the methionine tRNAs from *E.coli* (13).

A fourth pattern of MTSBS (residues 507−512) is conserved in the sequences of the MTS from *E.coli* (530−537) and yeast (mitochondrial: 536−541: cytoplasmic: 662−667) . In the four enzymes, these patterns are found in the same relative position with respect to the KMSKS regions. In the 3D-structure of the *E.coli* MTS, the residues 530−537 are part of a C-arm folding back towards the N-terminal domain (14). This region, which overhangs the active site crevice, is thought to guide the 3′-acceptor arm of the tRNA towards the active centre (21).

Three additional highly conserved segments are observed in the four *E.coli, B.stearothermophilus*, and yeast (cytoplasmic and mitochondrial) MTS (regions 230, 260 and 320 in the MTSBS sequence; Figure 6). The 320 region aligns with the 355 region of the *E.coli* enzyme which makes part of the junction between the N- and C-terminal domains in the 3D model. Interestingly, each of the two other segments contain one Trp residue. In the cases of *E.coli* and *B.stearothermophilus* enzymes, binding of methionine induces an important proteic fluorescence change (13, 35). Therefore, it is tempting to speculate that these conserved regions might be present in the methionine binding site of MTS.

A final remark concerns the difference in length between the two methionyl-tRNA synthetase sequences (Figure 6). Clearly, the larger size of the *E.coli* enzyme is due to the presence in its sequence, between residues 100 and 200, of well-defined stretches of additional amino acids rather than of a random distribution of extra amino acids. According to the 3-D structure of the *E.coli* enzyme (14), these stretches are located in loop III and are not likely to be involved in the folding of the active site.

To conclude, the *E.coli* and *B.stearothermophilus* methionyl-tRNA synthetases appear to share many common features at the level of structure−function relationships, despite the low degree of sequence homology. Consequently, those sequences which are conserved within the two enzymes may be suspected to be particularly significant in the expression of the specificity of action of methionyl-tRNA synthetase. This prediction is currently being studied using site-directed mutagenesis.

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