The Amber Codon in the Gene Encoding the Monomethylamine Methyltransferase Isolated from *Methanosarcina barkeri* Is Translated as a Sense Codon*

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Each of the genes encoding the methyltransferases initiating methanogenesis from trimethylamine, dimethylamine, or monomethylamine by various *Methanosarcina* species possesses one naturally occurring in-frame amber codon that does not appear to act as a translation stop during synthesis of the biochemically characterized methyltransferase. To investigate the means by which suppression of the amber codon within these genes occurs, MtmB, a methyltransferase initiating metabolism of monomethylamine, was examined. The C-terminal sequence of MtmB indicated that synthesis of this *mtmB1* gene product did not cease at the internal amber codon, but at the following ochre codon. Antibody raised against MtmB revealed that *Escherichia coli* transformed with *mtmB1* produced the amber termination product. The same antibody detected primarily a 50-kDa protein in *Methanosarcina barkeri*, which is the mass predicted for the amber readthrough product of the *mtmB1* gene. Sequencing of peptide fragments from MtmB by Edman degradation and mass spectrometry revealed no change in the reading frame during *mtmB1* expression. The amber codon position corresponded to a lysyl residue using either sequencing technique. The amber codon is thus read through during translation at apparently high efficiency and corresponds to lysine in tryptic fragments of MtmB even though canonical lysine codon usage is encountered in other *Methanosarcina* genes.

The methylamines are significant precursors for methane formation in marine and brackish environments (5). Recent studies have demonstrated that a considerable number of proteins and genes in *Methanosarcina* species are dedicated to the utilization of methylamines. Methanogenesis from methylamines requires methylation of coenzyme M (CoM) prior to reduction of the methyl group to methane. *In vitro* reconstitution with purified proteins has shown that CoM methylation is initiated with one of three different methylamine methyltransferases that methylate a cognate corrinoid protein (Fig. 1A). The TMA methyltransferase (MttB) and cognate corrinoid protein (MttC) are specific for TMA (6); the DMA methyltransferase (MtB) and cognate corrinoid protein (MtB) are specific for DMA (7); and the MMA methyltransferase (MmmB) and cognate corrinoid protein (MmmC) are specific for MMA (8). Each of the methylated cognate corrinoid proteins can be demethylated by the methylamine-CoM methylase (MtbA), yielding methyl-CoM.

The genes encoding TMA, DMA, and MMA methyltransferases were identified by the N-terminal sequences of the isolated methyltransferases (9, 10) (Fig. 1B). The gene sequences reveal that the TMA, DMA, and MMA methyltransferases are not homologous proteins. However, the genes encoding each of the methylamine methyltransferases share the common feature of a single internal amber codon. The amber codons, if read as stop codons, would result in truncated proteins of 23 kDa (MtmB), 38 kDa (MtbB), and 32 kDa (MtB). For each gene, a downstream ochre or opal stop codon follows the in-frame amber codon. If translation stopped at this second canonical stop codon, each gene would produce protein products of ~50 kDa, the approximate molecular mass of the isolated and characterized TMA, DMA, or MMA methyltransferase polypeptide. These data indicate that the internal amber codons of the methylamine methyltransferase genes do not cease translation when the 50-kDa methyltransferases are produced.

Isolation of the TMA, DMA, and MMA methyltransferases resulted in the purification of a single protein for each activity (6–8). However, multiple and nearly identical copies of the genes encoding the DMA and MMA methyltransferases have been found in the genome of *M. barkeri* strain MS (Fig. 1B). The three *mtbB* genes are predicted to encode proteins with an average of 95% identity at the deduced amino acid level (9). Two MMA methyltransferase gene copies are present in *M. barkeri* MS. These have been designated *mtmB1* (formerly designated *mtmB* (10), GenBank™/EBI accession number AF013713) and *mtmB2* (GenBank™/EBI accession number...

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§ The abbreviations used are: TMA, trimethylamine; DMA, dimethylamine; MMA, monomethylamine; CoM, coenzyme M; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight.

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AF230870\(^2\) and share 98% identity at the deduced amino acid level. Both genes have an in-frame amber codon at codon 202 and a downstream TAA stop codon at position 458. Termination at the second stop codon would result in a 50-kDa gene product, the mass of the isolated MMA methyltransferase (MtMB).

The internal amber codons of these genes were first found by directed sequencing of the methylamine methyltransferase genes of *M. barkeri* strains MS and NIH and *Methanosarcina thermophila* (9, 10). Recently, genomic sequencing of *Methanosarcina mazei* at the Göttingen Genomic Institute\(^3\) and *M. barkeri* Fusaro at the Department of Energy Joint Genome Institute confirmed that all TMA, DMA, and MMA methyltransferase genes within these organisms also contain a single in-frame amber codon. Such genus-level re-coding events, in which the termination function of a naturally occurring canonical stop codon is suppressed, remain relatively rare, but have been now documented in a variety of different organisms (11).

In the most extreme examples, such as *Mycoplasma* species, some protists, and mitochondria of many species, a canonical stop codon serves as a general sense codon (12). However, in the ~200 sequenced genes of *Methanosarcina* species in the GenBank\(^4\)/EBI Data Bank, no gene other than the methylamine methyltransferase genes has been observed to utilize internal amber codons as sense codons. The methylamine methyltransferase genes themselves possess only a single internal amber codon in each gene (Fig. 1B).

Several means by which a naturally occurring internal stop codon is suppressed have been identified. In some cases, the stop codon is avoided by frameshifting (13, 14) such as observed for the retroviral gag-pol genes (15), the gene encoding *Escherichia coli* release factor II (16), and the genes encoding ornithine decarboxylase antienzymes in eucaryal species (17). Frameshifts can also occur in which the sequence containing a contiguous sequence, such as with T4 gene 60 (18). Of course, a naturally occurring stop codon can also be suppressed with a tRNA whose anticodon can base pair with the stop codon. Some eucaryotic tRNA species that suppress naturally occurring stop codons also decode normal sense codons (19, 20). In contrast, dedicated tRNA species are involved in decoding UGA as selenocysteine in all three domains (21).

Aside from the UGA encoding of selenocysteine (22), the suppression of a naturally occurring in-frame canonical stop codon had not been previously observed in genes of any member of the methanogens or, indeed, any other Archaea. Thus, the manner in which the in-frame amber codons of the methylamine methyltransferase are suppressed has been unknown. The single in-frame amber codons of the methylamine methyltransferase genes are given added interest by occurring in non-homologous genes with analogous roles in initiating metabolism of a methylamine. The relative abundance of methanogen catabolic proteins such as the methyltransferases (6–8) encoded by these genes suggests that an efficient amber codon readthrough mechanism would be needed to facilitate expression.

To examine the extent and manner of readthrough of the internal amber codon of the methylamine methyltransferase genes, the *mtmB1* gene product (MtMB) was examined. The results indicate that the internal amber codon of the gene encoding this protein is not suppressed by frameshifting, but that the amber codon position in the gene corresponds to lysine in the protein product.

**EXPERIMENTAL PROCEDURES**

**Cell Cultivation and Extracts—** *M. barkeri* MS cells were grown on 80 mM MA or sodium acetate in a phosphate-buffered medium (23) and harvested 7 days after inoculation. Cell extracts were prepared by lysis with a French pressure cell as described previously and frozen at 70 °C prior to use (24).

**Tryptic Digests of MtMB—** The MtMB protein used throughout this study was purified as described by Burke and Krzycki (8) from cells grown on MMA. Protein was assayed using the bicinchoninic acid assay (25) with bovine serum albumin as the standard. Protein purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) following the method of Laemmli (26), followed by staining with Coomassie Brilliant Blue. The protein was carboxymethylated after reduction of 86 μM MtMB with 14.2 mM diethiothreitol in 7.1 mM Tris buffer (pH 8.0) for 20 min at 50 °C under N\(_2\). After cooling to room temperature, iodoacetate (53.8 mM) was added. If radiolabeled peptides were desired, \(^{13}C\)iodoacetate (ICN Pharmaceuticals, Costa Mesa, CA) was used at a specific radioactivity of 13.4 μCi/mmol. After overnight incubation, the mixture was adjusted to a final concentration of 6.6 mM urea, 0.33 mM ammonium bicarbonate, and 17.6 mM diethiothreitol. The mixture was then incubated at 50 °C for 20 min prior to addition of 29 mM fresh iodoacetate. After a final 45 min incubation at 37 °C, the carboxymethylated protein was exchanged into 0.4 mM ammonium bicarbonate buffer containing 2 mM urea and digested with 33% (w/v) trypsin (Roche Molecular Biochemicals) for 20 h at 37 °C. The digest was analyzed by reverse-phase HPLC analysis using a 25 × 0.46-cm C\(_{18}\) column (Rainin Instrument Co., Inc., Woburn, MA). The column was eluted with a 52.5-ml gradient beginning with 0.06% (v/v) trifluoroacetic acid and 1.2% (v/v) acetonitrile and ending with 0.052% trifluoroacetic acid and 80% acetonitrile at a flow rate of 0.5 ml/min and monitored at 210 nm. Radiolabeled fragments were identified using the on-line radioactivity detector described previously (24).
N- and C-terminal Sequencing—The intact MtmB protein or HPLC-purified tryptic fragments of MtmB were sequenced by automated Edman degradation using an Applied Biosystems Model 473A sequencer. For C-terminal sequencing, protein was loaded onto an Applied Biosystems ProSorb cartridge. The polyvinylidene difluoride membrane from the cartridge was then analyzed using an Applied Biosystems Model 494C sequencer.

Purification of Anti-MtmB Antibodies—Purified MtmB was electrophoresed through an SDS-15% polyacrylamide gel, and the excised MtmB was used to raise rabbit antibodies by Harlan Bioproducts Inc. (Cincinnati, OH). The polyclonal antibodies were purified with an affinity column. Purified MtmB was bound to an N-hydroxysuccinimide-activated Sepharose column (Amersham Pharmacia Biotech) following the manufacturer’s protocol. Immune antisera was applied to the column, which was equilibrated with 75 mM Tris buffer (pH 8.0), and the antibody was eluted with 100 mM glycine buffer (pH 3.0) and 500 mM NaCl and collected in 1-ml fractions neutralized with 0.4 ml of 1 M Tris buffer (pH 8.0). Immunoblots of SDS-polyacrylamide gels were prepared as described previously (27) using 4-chloro-1-naphthol as substrate for the horseradish peroxidase-linked sheep anti-rabbit secondary antibodies (Sigma).

Construction of pCJ09—Oligonucleotide primers were designed that added NdeI and EcoRI restriction sites to the 5′- and 3′-coding sequences of mtmB1. These primers were used to amplify, from a subclone of λ-SAB (10), a fragment comprising the entire coding region of mtmB1 extending from the ATG start codon through the internal amber codon to the following TAA codon (nucleotide +1377 relative to the starting nucleotide). The polymerase chain reaction product was cloned into pGEM-T (Promega, Madison, WI) following the manufacturer’s protocol. The complete mtmB1 gene was then excised with NdeI and EcoRI and ligated into similarly digested pET-17b (Novagen, Madison, WI) to create pCJ09. The DNA sequence of each mtmB clone was unchanged as confirmed using an ABI Prism Model 310 automated sequencer (PerkinElmer Life Sciences).
Expression of mtmB1 was induced with isopropyl-β-D-thiogalactopyranoside in E. coli BL21(DE3) pLysS transformed with pCJ09. The cells were lysed by sonication, solubilized with 1.2% SDS, and separated by SDS-PAGE on a 12.5% acrylamide gel prior to immunoblotting.

Mass Spectrometry—A tryptic fragment of MtmB that spanned the domain encoded by the region of mtmB1 containing the UAG residue was further digested with endoproteinase Glu-C (28). The peptides were separated by reverse-phase HPLC, and the masses of individual peptides were determined using electrospray ionization spectra taken with a Perceptive Biosystems Voyager-DE STR instrument. Samples were applied to the target using 4-hydroxy-a-cinnamic acid as the matrix. The sequence of a peptide fragment from a trypsin/Glu-C digest of MtmB encoded by the region surrounding the internal amber codon of mtmB1 was determined using Edman degradation of the N- and C-terminal sequences of the intact MtmB protein. A 42-kDa protein was detectable with anti-MtmB antibodies in purified MtmB preparations as well as in extracts of M. barkeri grown on MMA, but not acetate. Some of these same bands were also visible on immunoblots with higher amounts of MtmB, although they were undetectable on Coomasie Blue-stained gels of MtmB (Fig. 2A), indicating that they were relatively minor proteins compared with the 50-kDa MtmB protein. A 42-kDa protein was detectable with anti-MtmB antibodies in purified MtmB preparations as well as in extracts of M. barkeri. The intensity of the 42-kDa protein band was noted to increase in extracts that were repeatedly frozen and thawed (data not shown). This behavior, along with the 42-kDa mass, indicated that this band is a degradation product of the 50-kDa MtmB protein, rather than the amber termination product of the mtmB genes.

RESULTS

Readthrough of the Amber Codon of mtmB1 Is Required for Synthesis of MtmB—Cells of M. barkeri grown on MMA were harvested shortly after the onset of stationary phase, and the MMA methyltransferase (MtmB) was isolated according to an established protocol (8). This yielded a homogeneous preparation of the 50-kDa enzyme (Fig. 2A) as judged by Coomasie Blue-stained SDS-polyacrylamide gel. The mtmB1 and mtmB2 gene products are predicted to have the N-terminal sequences MTFRKSF and MTRFKYF, respectively. The N terminus of purified MtmB was determined to be TFRKSF. The fifth cycle of the Edman degradation revealed no tyrosyl residue, and only a seryl residue was detected.

The C terminus of MtmB was sequenced and found to be DLGLVF. This is the sequence predicted for both the mtmB1 and mtmB2 gene products if translation ends at the stop codon (TAA, codon 459) following the in-frame amber codon mtmB1. Termination at the TAA codon during expression of mtmB1 should produce a protein of 50 kDa. These results indicate that the isolated 50-kDa MtmB protein is produced from mtmB1 with the internal amber codon not serving as a translation stop and that, instead, translation termination occurs at the following TAA codon.

The Amber Termination Product of mtmB1 Is Expressed in E. coli—The M. barkeri mtmB1 gene, including the internal amber codon and the following TAA stop codon, was cloned into an expression vector, yielding pCJ09. E. coli transformed with pCJ09 produced a 27-kDa polypeptide that increased upon isopropyl-β-D-thiogalactopyranoside induction (Fig. 2A). This protein was not observed in the same host strain that had been transformed with pET-17b lacking the mtmB1 insert. The predicted size of the amber codon termination product of mtmB1 is 23 kDa. To confirm that the recombinant protein was the mtmB1 amber termination product, cell extracts of E. coli carrying pCJ09 were separated by SDS-PAGE, blotted onto polyvinylidene difluoride membrane, and probed with polyclonal antibody raised against the 50-kDa MtmB protein (Fig. 2B). The inducible 27-kDa protein was readily detectable with anti-MtmB antibody. Following induction of mtmB1, the predominant 27-kDa polypeptide was excised from the gel following SDS-PAGE of the E. coli extract, and the C terminus was determined to be GV. This corresponds to the C-terminal sequence predicted for the amber codon termination product of mtmB1.

The 50-kDa MtmB Protein Is the Predominant Product of mtmB Genes Produced in M. barkeri—The antibody raised against 50-kDa MtmB was used to examine cell extracts of M. barkeri following growth on either MMA or acetate to determine the relative abundance of the 50-kDa MtmB protein and any putative amber termination products produced from mtmB genes. By far the most intense band detectable on immunoblots of extracts of M. barkeri grown on either acetate or MMA was 50-kDa MtmB, as evidenced by comigration with the purified MtmB protein (Fig. 2B). Minor bands corresponding to proteins smaller than 50 kDa were also detectable on immunoblots of extracts of M. barkeri grown on MMA, but not acetate. Some of these same bands were also visible on immunoblots with higher amounts of MtmB, although they were undetectable on Coomasie Blue-stained gels of MtmB (Fig. 2A), indicating that they were relatively minor proteins compared with the 50-kDa MtmB protein. A 42-kDa protein was detectable with anti-MtmB antibodies in purified MtmB preparations as well as in extracts of M. barkeri. The intensity of the 42-kDa protein band was noted to increase in extracts that were repeatedly frozen and thawed (data not shown). This behavior, along with the 42-kDa mass, indicated that this band is a degradation product of the 50-kDa MtmB protein, rather than the amber termination product of the mtmB genes.

Three minor bands of 26, 28, and 30 kDa were also detected in extracts of M. barkeri grown on MMA probed with anti-MtmB antibodies. Since these proteins migrated roughly the same distance as the E. coli mtmB1 amber termination product, they may represent amber termination products of the mtmB genes. Immunoblots of serial dilutions of the extract demonstrated that the intensity of the immunoblot signal corresponding to the 50-kDa MtmB protein began to saturate before the three putative mtmB amber termination products were even detectable (Fig. 2C), making estimates of relative amounts difficult. With the lowest amount of cell extract in which the putative amber termination products were seen (20 μg of total protein), image analysis indicated that the total intensity of these three protein bands represented ∼3.5% of the total signal from 50-kDa MtmB. Although this overestimates the signal intensity of the putative amber termination products relative to the 50-kDa MtmB signal, this result does indicate that relatively small amounts of any potential MtMB amber termination products were present in the M. barkeri cells.

Internal Sequencing of the mtmB1 Gene Product (MtM)—In the absence of any frameshift events, the tryptic fragment of MtmB corresponding to the region of mtmB1 surrounding the amber codon is predicted to possess three cysteine residues. To identify this peptide for sequencing by Edman degradation,

MTFRKSFDYFDRKVEGKCTQDDWLMKIPMAMELKQKYLDFK
GEFIPITDKDMMEKLKAGFEMMLCGYCTDHRKYTEDWADNVQ
KEFVLGTRGDAVNVKRSGVDKAKPQGVGGPTSPISDEVFMVPVMHSYALE
KEVDTIVNMTSVMRKSPIKSPYEVLAAKETTRLKACAMAGRPNGM

|Fig. 5. Amino acid sequence of the predicted gene product of the mtmB1 gene. The boldface residues were sequenced by Edman degradation of the tryptic fragments of purified Mtb. The N- and C-terminal sequences of the intact Mtb protein are also indicated. The lysine residue corresponding to the location of the amber codon within mtmB1 is boldface and boxed. |
sin-digested 14C-carboxymethylated protein was then separated by reverse-phase HPLC and analyzed by Edman degradation (Fig. 5) had N-terminal sequences identical to those of tryptic fragments predicted from mtmB1. These results confirmed that no frame-shifting or translational bypassing event was required to express MtmB from mtmB1. Internal sequencing of the MtmB protein also provided further evidence that the isolated MtmB protein was produced from mtmB1 rather than mtmB2. Two internal peptides were identified (CTQDDGW and LVSL) whose sequences were identical to the N termini of predicted tryptic fragments of the mtmB1 gene product, whereas the boldface residues are replaced by Leu and Ala, respectively, in the predicted product of the mtmB2 gene. In addition, one tryptic fragment of MtmB had the N-terminal sequence GNSDI, which would be predicted to derive only from the mtmB1 gene product since, immediately preceding this sequence, a Thr residue rather than Lys is predicted for the mtmB2 gene product.

Mass Spectrometric Analysis of a Peptide Containing the UAG-encoded Residue—The MtM tryptic fragment identified above spanned the corresponding amber codon region of mtmB1, but was too large for sequence analysis by mass spectrometry and was also 14C-labeled. To produce a smaller non-radioactive fragment, the tryptic digest was repeated with [12C]iodoacetate-treated protein and separated by HPLC. With this digest, the tryptic fragment beginning with NACAMAGRPGMGV was not identified, but eluting at almost the same position from the reverse-phase column was a peptide generated by trypsin cleavage following Arg196, rather than Lys189, whose N terminus began with PGMGV (see Fig. 5). Edman degradation of this peptide confirmed the sequence predicted from mtmB1 before and after the amber-encoded residue at position 202 in MtmB, which again was identified as lysine.

The HPLC fraction containing this peptide was then further digested with endoproteinase Glu-C, which cleaves following either Asp or Glu. The digest was further purified by reverse-phase HPLC. The masses of the eluting peptides were screened by MALDI-TOF, and a peptide with a mass/charge ratio of 2017.62 was detected (Fig. 6A). This mass matches that predicted for the peptide PGMGVKGPETSLSAQGNISAD. This sequence corresponds to a peptide fragment predicted by the mtmB1 sequence if the reading frame surrounding the UAG codon is maintained and the amber codon itself encodes lysine. The peptide was further analyzed by tandem mass spectrometry (Fig. 6B). The sequence inferred from the peptide mass was confirmed by the masses determined for the individual residues. The residue corresponding to the position encoded by the UAG codon in the peptide had a mass of 128.1 Da. This corresponds to the mass expected if the amber codon of mtmB1 directs incorporation of lysine into MtmB.

**DISCUSSION**

An internal amber codon had not been found to be a common feature of a class of genes from any archaeal species until the genes encoding the TMA, DMA, and MMA methyltransferases were described. Here, the purified MMA methyltransferase MtmB was examined as the product of a representative methylamine methyltransferase gene. The residues directly N- and C-terminal to the UAG-encoded position are those predicted by the reading frame of mtmB1. The amino acid residue found at the position corresponding to the UAG codon was identified as a lysyl residue by both Edman degradation and mass spectrometry.
The correspondence of UAG to lysine in proteolytic fragments of the MMA methyltransferase is surprising given that otherwise normal use of AAA and AAG codons in almost equal proportion is observed in the genes from Methanosaeta species deposited in the GenBank/EBI Data Bank. This assignment is also notable in that no peptides were identified from MtmB digests in which this lysine codon served as a tryptic cleavage site. However, digestion with trypsin did not proceed to completion in these digests, as evidenced by a sequenced peptide in which trypsin cleavage after an Arg residue did not occur. In addition, the Gly and Pro residues following the UAG-encoded lysine could negatively influence trypsin cleavage. The sequencing of isolated tryptic fragments also cannot exclude a labile modification of the amber-encoded lysine in the intact protein. In any case, these results indicate that, during translation of mtmB1 resulting in synthesis of 50-kDa MtmB, the internal amber codon encodes an amino acid residue. This eliminates other potential mechanisms by which the amber codon might have been circumvented during expression of full-length MtmB, such as frameshifting with or without the translation of noncontiguous sequences.

A potential complication in this analysis existed. Two nearly identical genes (mtmB1 and mtmB2) that could encode the characterized MtmB protein and that contain an internal amber codon at corresponding positions are found in the genome of M. barkeri MS. However, sequencing of the isolated MtmB protein provided no indication of the presence of the mtmB2 gene product and was entirely as predicted for the mtmB1 gene product. This result was consistent with recent results from our laboratory indicating that the mtmB1 transcript is abundant relative to the mtmB2 transcript. However, even in the event that the isolated MtmB product was a mixture of both gene products, the conclusion that the amber codon corresponds with lysine in proteolytic fragments of the MMA methyltransferase would remain valid. The mtmB genes have identical sequence surrounding and including the in-frame amber codon, and the following ochre codon is also at the same position in both genes.

An alternative explanation for the apparent readthrough of an amber codon during synthesis of MtmB is that a gene nearly identical to mtmB1 in the genome has a lysine codon replacing the internal amber codon, but otherwise exactly matches the sequenced portions of MtmB (Fig. 5). However, no evidence has been found for such a gene. Extensive probing of restricted genomic DNA has revealed only DNA fragments corresponding to mtmB1 and mtmB2 in the genome of M. barkeri MS. Genomic sequencing of M. mazei has revealed two copies of mtmB genes. BLAST searches of the preliminary sequence data for the M. barkeri Fusaro genome on the server of the Department of Energy Joint Genome Institute (www.jgi.doe.gov) revealed three mtmB1 homologs. All mtmB homologs found in both genomes possess the in-frame amber codon at the position noted in M. barkeri MS mtmB1 and are predicted to encode 50-kDa MMA methyltransferases if their internal amber codons are translated.

E. coli transformed with mtmB1 produced an inducible 27-kDa product with the C terminus predicted for the mtmB1 amber termination product and that reacted with anti-Mtb antibody. This served as a control for detection of the mtmB1 amber termination product within M. barkeri, but only trace amounts of M. barkeri proteins near the size of the mtmB1 amber termination product produced in E. coli were found. The identification of these products as actual mtmB amber termination products is by no means certain. However, their low relative abundance clearly indicates that relatively little of even potential amber termination products exist in the cell relative to the amber readthrough product. Again, the presence of the nearly identical mtmB1 and mtmB2 genes in the genome is a potentially complicating factor, but since the two gene products are 98% identical, the polyclonal antibody raised against MtmB should detect both full-length and amber termination products from either gene. These results are thus consistent with suppression of the amber codon within the mtmB genes occurring at high efficiency in M. barkeri. It remains possible that high amounts of amber codon termination products of the mtmB genes are produced, but are degraded by the cell. However, high efficiency readthrough of the amber codon would be consistent with the abundance of Mtb, which is >2% of soluble protein in M. barkeri (8). A markedly lower efficiency readthrough of the amber codon, such as the 5% readthrough of amber codons reported for the retroviral Gag-Pol protein (29), would result in production of a large amount of cellular protein as the mtmB amber codon termination products.

Thus far, internal amber codons have been found only in the genes encoding the TMA, DMA, and MMA methyltransferases in methanogenic Archaea. Twenty-one examples of these methyamine methyltransferase genes have been now sequenced, and all maintain the single in-frame amber codon. Assuming that the in-frame amber codon encodes lysine in these methyltransferases, this argues for a powerful selection against the simple point mutation required to convert the amber UAG codon to a lysine codon (AAG or AAA). What this selective pressure might be is as yet unknown, but it is reasonable to postulate a regulatory or catalytic function related to the common role of the encoded proteins in initiating the assimilation and catabolism of methylamines.

Scenarios can be envisioned in which the amber codon is required for synthesis of catalytically active MtmB. For example, pausing at the amber codon during translation may allow proper folding of MtmB, or the amber codon could be involved in a modification of a residue needed for full catalytic activity. It must be stressed that the current results do not eliminate the possibility of a labile modification near or even on the amber-encoded lysine residue, which might be lost during the acidic conditions employed for purification of the peptides sequenced here. Alternatively, the in-frame amber codon of the methyamine genes may represent a regulatory device in which the encoding of the diamino acid lysine by an amber codon acts in an as yet unelucidated manner to control metabolism of methyamine substrates by controlling methyamine methyltransferase gene expression. Regulatory functions have been demonstrated for the in-frame stop codons in the genes encoding ornithine decarboxylase antienzymes (17) as well as release factor II in E. coli (30–32).

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REFERENCES
1. Woese, C. R., Kandler, O., and Wheelis, M. L. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 4576–4579
2. Boone, D. R., Whitman, W. B., and Rouvière, P. (1993) in Methanogenesis: Ecology, Physiology, Biochemistry, and Genetics (Ferry, J. G., ed.), pp. 35–80, Chapman and Hall, Inc., New York
3. Tauer, R. K. (1998) Microbiology 144, 2377–2406
4. Ferry, J. G. (1999) FEMS Microbiol. Rev. 23, 13–38
5. King, G. M. (1984) Geomicrobiol. J. 3, 276–301
6. Ferguson, D. J., Jr., and Kryzick, J. A. (1997) J. Bacteriol. 179, 846–852
7. Ferguson, D. J., Jr., Gorallova, N., Grahame, D. A., and Kryzick, J. A. (2000) J. Biol. Chem. 275, 29053–29060
8. Burke, S. A., and Kryzick, J. A. (1997) J. Biol. Chem. 272, 16570–16577
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9. Paul, L., Ferguson, D. J., and Krzycki, J. A. (2000) *J. Bacteriol.* 182, 2520–2529
10. Burke, S. A., Lo, S. L., and Krzycki, J. A. (1998) *J. Bacteriol.* 180, 3432–3440
11. Gesteland, R. F., and Atkins, J. F. (1996) *Annu. Rev. Biochem.* 65, 741–768
12. Osawa, S., Jukes, T. H., Watanabe, K., and Muto, A. (1992) *Microbiol. Rev.* 56, 229–264
13. Alam, S. L., Atkins, J. F., and Gesteland, R. F. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 14177–14179
14. Farabaugh, P. J. (1996) *Microbiol. Rev.* 60, 103–134
15. Jacks, T., Madhani, H. D., Mastarz, F. R., and Varmus, H. E. (1998) *Cell* 55, 447–458
16. Craigen, W. J., and Caskey, C. T. (1986) *Nature* 322, 273–275
17. Ivanov, I. P., Gesteland, R. F., and Atkins, J. F. (2000) *Nucleic Acids Res.* 28, 3185–3196
18. Herr, A. J., Atkins, J. F., and Gesteland, R. F. (2000) *Annu. Rev. Biochem.* 69, 343–372
19. Geller, A. L., and Rich, A. (1980) *Nature* 283, 41–46
20. Feng, Y. X., Copeland, T. D., Oroszlan, S., Rein, A., and Levin, J. G. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 8860–8863
21. Commans, S., and Bock, A. (1999) *FEMS Microbiol. Rev.* 23, 335–351
22. Rother, M., Wilting, R., Commans, S., and Bock, A. (2000) *J. Mol. Biol.* 299, 351–358
23. Krzycki, J. A., Mortenson, L. E., and Prince, R. C. (1989) *J. Biol. Chem.* 264, 7217–7221
24. Cao, X., and Krzycki, J. A. (1991) *J. Bacteriol.* 173, 5439–5448
25. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) *Anal. Biochem.* 150, 76–85
26. Laemmli, U. K. (1970) *Nature* 227, 680–685
27. Ferguson, D. J., Jr., Krzycki, J. A., and Grahame, D. A. (1996) *J. Biol. Chem.* 271, 5189–5194
28. Taylor, J. M., Mitchell, W. M., and Cohen, S. (1974) *J. Biol. Chem.* 249, 2188–2194
29. Wills, N. M., Gesteland, R. F., and Atkins, J. F. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 6991–6995
30. Craigen, W. J., Cook, R. G., Tate, W. P., and Caskey, C. T. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 3616–3620
31. Persson, B. C., and Atkins, J. F. (1998) *J. Bacteriol.* 180, 3462–3466
32. Stadtman, T. C. (1996) *Annu. Rev. Biochem.* 65, 83–109
