Use of *bgaH* as a reporter gene for studying translation initiation
in the archaeon *Haloferax volcanii*

by

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**Abstract:**

The *bgaH* gene isolated from *Haloferax lucentensis* codes for β-galactosidase. To study the function of initiator tRNAs in translation initiation in *Haloferax volcanii*, the initiator AUG codon of the *bgaH* gene was mutated to UAG, UAA, UGA, and GUC. Four different *H. volcanii* initiator tRNA derived mutants with complementary anticodons were also made. When plasmids carrying the *bgaH* reporter and mutant initiator tRNAs were coexpressed in *H. volcanii*, the UGA and GUC decoding tRNAs were aminoacylated, but functional β-galactosidase was produced only in the presence of the latter tRNA. This result confirms that translation can initiate with some alternative codons, but suggests that the amino acid attached to the tRNA also plays a role. It is unknown if leaderless transcripts will have similar requirements, therefore mutant *bgaH* reporters lacking 5' untranslated regions were also generated.

I also describe modifications of the *bgaH* reporter for studying suppression of termination codons in *H. volcanii*. The serine codon at position 184 of the *bgaH* gene was mutated to the termination codons UAA and UAG. *H. volcanii* serine tRNA derived suppressor tRNAs with complementary anticodons were also generated. These suppressor tRNAs should allow a study of the requirements for suppression of UAG and UAA codons in *H. volcanii*, in particular the question of whether suppressors of the UAA codon can also suppress the UAG codon in archaea.

*H. volcanii* WFD11 used as the host does not have any endogenous β-galactosidase. I have shown that extracts made from *H. volcanii* transformants can be used to assay for β-galactosidase using either O-Nitrophenyl-β-galactoside or Beta-Glo reagent as a substrate. This latter assay couples the D-Luciferin product of cleavage of 6-O-β-galactopyranosyl-luciferin by β-galactosidase to the more precise and sensitive luciferase assay.

Since little is known about translation in archaea, future work will involve modifying identity elements in the initiator tRNA to study their requirements in both initiation and elongation in archaea.

Thesis Supervisor: Uttam L. RajBhandary

Title: Lester Wolfe Professor of Molecular Biology

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## Table of Contents

**Introduction:**

The "Central Dogma" of molecular biology describes how genetic information, encoded in DNA, is transcribed to messenger RNA (mRNA) and then translated to protein. Translation occurs on a large protein-RNA complex called the ribosome. It is on this machine that each three nucleotide codon directs the addition of amino acids into the nascent polypeptide chain through interactions with adapter molecules, called transfer RNA (tRNA).

Each tRNA is specifically aminoacylated (or "charged") by an aminoacyl-tRNA synthetase (aaRS). These enzymes attach an amino acid to the 3' end of the tRNA, which then carries it to the ribosome. The tRNA also has a three nucleotide anticodon sequence which forms base pairs with specific codons in the mRNA. During translation, the ribosome ensures that only correct codon / anticodon pairs are made, and then covalently links the amino acid attached to the tRNA to the growing polypeptide chain using the peptidyl transferase activity. By repeating this process until a stop signal is reached, the mRNA is translated into a functional protein.

Translation can be divided into three phases; initiation, elongation, and termination. The first step, initiation, involves assembly of the ribosome, mRNA, and the initiator tRNA. Aminoacylated initiator tRNA is bound to the ribosomal P site and pairs with the complementary start codon, which is almost always AUG. Elongation occurs with aminoacylated tRNAs entering the ribosomal A site and pairing with the next codon, matches are found, and the amino acid is transferred. Termination usually occurs when one of the stop codons (UAG, UAA, and UGA) is encountered. The protein is then released from the tRNA with the help of release factors and the mRNA and ribosome dissociate.

Before describing how translation differs in the three major domains of life, I first provide an overview of the relatively newly discovered Archaea. Traditionally, organisms had been classified into two domains of life, the eukaryotes and the prokaryotes, based respectively on the presence or lack of a nucleus. The term prokaryote had been synonymous with bacteria, but work by Carl Woese indicated that the domain should be further divided.

While comparing the conserved small 16S rRNA sequences, bacteria and eukarya grouped as expected, but a third group was also found (Woese, 1977). These organisms were prokaryotic, in that they lacked a nucleus, but based on comparison of ribosomal RNA sequences they appeared distinct from the bacteria. Further evidence has shown that the group, now called

Archaea, share many similarities to the Eukaryotes. For instance, they have histone-like proteins and RNA polymerases that have a similar number of subunits (Coulson, Touboul , and Ouzounis, 2007). However, the archaea also have unique properties, such as the composition of their cell membranes. Most phylogenetic trees now depict the archaea as an evolutionary link between the other two domains, notwithstanding a significant amount of horizontal gene transfer.

As the catalytic mechanism of translation is conserved throughout all three domains this process is considered to be ancient, present in the putative last universal common ancestor (LUCA) (Londei, 2005). In fact, as the catalytic core of translation is RNA based, arguments have been made that this ribozyme present in the LUCA is a remnant of an RNA world that preceded it (Polacek and Mankin, 2005). Therefore, studying this basic process will give insights into how life has evolved in the three domains.

Understanding translation in the archaea should first be approached by considering its similarities to the other two domains. A recent review by Paola Londei (Londei, 2005) provides an overview of these features which are summarized below.

In all three domains, translation begins with an AUG start codon. The methionine initiator tRNA required for this step is unique in that it has many special features in its sequence and it is directly recruited to the ribosomal P site. In all organisms, it is believed that this occurs by recognition of identity elements in the tRNA's anticodon stem, however this has not been proven for the archaea (Stortchevoi, Varshney, and RajBhandary, 2003). Bacteria further modify the attached methionine by formylating it, a step not present in archaea or in eukaryotes (White and Bayley, 1972).

To determine the correct start codon, bacterial ribosomes recognize the Shine-Dalgarno (SD) sequence approximately 7-13 nucleotides upstream of the AUG (Shine and Dalgarno, 1975). The consensus SD sequence, AGGAGG, pairs with a complementary sequence in the 3' end of the 16S ribosomal RNA. As bacterial mRNA is often polycistronic, containing multiple genes on the same mRNA transcript, translation can begin contemporaneously at several locations. On the other hand, translation of eukaryotic mRNA often proceeds from the first AUG of the transcript using a process called 'scanning' (Kozak, 1989). Eukaryotic mRNA is predominantly monocistronic; also it is further modified by addition of a cap structure at the 5' end and a poly(A) sequence at the 3' end.

Archaeal mRNAs are more bacterial like in that they are polycistronic and use SD sequences for translation of internal open reading frames. However, the transcripts are often leaderless, as a consequence the first AUG does not have an upstream SD sequence. This has led to the postulate that translation in the archaea either uses a bacterial type mechanism based on recognition of a SD sequence or a distinct leaderless mechanism (Tolstrup *et al.*, 2000). Further experiments have shown that ribosomes from all three domains can initiate translation from leaderless transcripts (Grill *et al.*, 2000). This finding has been used to infer that ribosomal recognition of leaderless transcripts is an ancient process present in the LUCA, and that only archaea continue to use this method as a primary means to initiate translation.

In contrast to the bacterial-like mRNA structure found in archaea, the accessory factors used in translation initiation are more eukaryotic-like. Bacteria utilize only 3 initiation factors; IF1, IF2, and IF3. Eukaryotic initiation requires approximately 10 initiation factors, many of which have homologues in the archaea. The only significant differences are the lack of cap binding proteins (eIF4F), eIF3 (which interacts with eIF4F), poly(A) binding protein, and a different method of a/eIF2 GDP↔GTP exchange (Kyrpides and Woese, 1998).

Translation initiation in the archaea can be thought of as a hybrid between bacteria and eukaryotes. Although biochemical studies have not been done to confirm the role of the eukaryotic-like initiation factors, it is clear that they function on mRNA transcripts that resemble those found in bacteria. How this is accomplished is unknown. The work presented here was aimed at analysis of how this process occurs in this poorly understood domain of life.

The immediate objective of my work was to investigate which of the anticodon sequence mutants of an archaeal initiator tRNA can be used to initiate protein synthesis from the *bgaH* reporter gene carrying corresponding mutations in the initiation codon (Figure 1). Toward this objective, I mutated the AUG initiation codon of the *bgaH* reporter gene to UAG, UAA, UGA and GUC. I also changed the CAU anticodon sequence of the *H. volcanii* initiator tRNA to sequences complementary to the above four codons. For expression in *H. volcanii*, the *bgaH* mutants were cloned into pMLH32 derived plasmids and the initiator tRNA mutants were cloned into pWL201 derived plasmids (Figure 2). Transformation of *H. volcanii* with these plasmids followed by assay for β-galactosidase in cell extracts showed that while all of the mutant tRNAs were expressed well, only two of them could be aminoacylated *in vivo*. Of the two, only the

mutant tRNA with the anticodon GAC could initiate protein synthesis using GUC as an initiation codon.

These results confirm previous findings using the bacterio-opsin gene in *H. salinarum* that translation in archaea can initiate with GUC but suggests that the amino acid attached to the tRNA also plays a role (Srinivasan, Krebs, and RajBhandary, 2006). Identification of the mutant initiator tRNA, which can initiate protein synthesis from a non-AUG codon, allows one to introduce additional mutations into potential identity elements in the initiator tRNA, for example the A1:U72 base pair at the end of the acceptor stem or the three consecutive G:C base pairs in the anticodon stem (Figure 3) and to study the effect of such mutations on function of the mutant tRNAs in initiation in *H. volcanii*. Other aspects of translation that this approach can be used to study are: nonsense suppression and initiation from leaderless mRNAs.

**Results and Discussion:**

**Construction of mutant initiator tRNAs**

The vector pUCsptProM has an *H. volcanii* $tRNA^{lys}$ promoter upstream of a modified *Saccharomyces cerevisiae* $tRNA^{pro}$ gene, followed by a transcription termination signal (as pUC302, Palmer and Daniels, 1994). To create the shuttle vector pWL201HvMet$_i$, V. Ramesh first replaced the yeast tRNA gene with the *H. volcanii* initiator tRNA gene, creating pUCsptHvMet$_i$ (Ramesh and RajBhandary, 2001). The entire expression cassette was then transferred into pWL201 (Figure 2A). This plasmid provides the DNA replication origins for maintenance in both *E. coli* and in *H. volcanii*. It also provides ampicillin resistance by the *bla* gene and mevinolin resistance by a mutant 3-hydroxy-3-methylglutaryl-CoA reductase gene (Nieuwlandt and Daniels 1990).

Site directed mutagenesis on the $tRNA_i^{Met}$ gene was used previously to generate tRNAs potentially capable of reading UAG or GUC as initiation codons ($tRNA_i^{Met}$UAG and $tRNA_i^{Met}$GUC respectively). I added to this set by creating the tRNAs that decode UAA and UGA, completing the set of potentially 'nonsense codon' reading initiator tRNAs. It was found that mutagenesis worked better when 5% DMSO was added, which reduces the secondary structure of the tRNAs (as ssDNA) during PCR and mutagenesis.

*H. volcanii* has a restriction barrier preventing it from being transformed by methylated DNA (Holmes, Nuttall, and Dyall-Smith, 1991). Therefore, after being confirmed by DNA sequencing (Sanger, Nicklen, and Coulson, 1977), shuttle vectors were passaged through either of two adenine methylation deficient *E. coli* strains, GM2163 or ER2925 (NEB). The latter strain also has the nonspecific Endonuclease I deleted (*endA*$^-$).

**The UGA decoding mutant initiator tRNA is aminoacylated *in vivo*.**

*H. volcanii* was transformed with plasmids expressing the mutant initiator tRNAs and total tRNA was extracted. This was then subjected to acid urea polyacrylamide gel electrophoresis followed by RNA blot hybridization and probed with a mixture of three oligonucleotides that did not target the tRNA anticodon. Because the probe also hybridized with the endogenous initiator tRNA it was impossible to discern whether the mutant tRNAs were charged (Figure 4)

The next strategy was to electrophorese each mutant tRNA on a separate gel, alongside tRNAs isolated from *H. volcanii* transformed with the empty vector pWL201. Then, radio-labeled probes specific to each mutant $tRNA_i^{Met}$ anticodon were used during Northern Blot analysis. The results of probing tRNAs isolated from cells transformed with pWLHvMetiUAG, UAA, UGA, and GUC are shown in Figures 5, 6, 7, and 8 respectively. Improvements in quality of data between B and A can be ascribed to the following changes that were introduced: The 5'-$^{32}P$ labeled probe / SSC mixture was filtered through a membrane filter to remove nonspecific radioactive spots throughout the membrane most likely due to the presence of particulate matter; also, the membrane for blotting and hybridization was changed from Hybond-$N^+$ (Amersham) to Nytran SPC (Whatman). These modifications led to more evenly distributed films and shorter exposure times.

In all cases, bands in *lanes 1* and *2* of B were more intense than *lanes 3* and *4*, indicating the mutant tRNAs were being expressed. The $tRNA^{ser}$ control was constant; indicating an equal amount of tRNA was loaded in each lane. $tRNA_i^{Met}$UAG was not significantly charged above background (Figure 5B, compare *lanes 1* and *3*), while $tRNA_i^{Met}$GUC was greater than 50% charged (Figure 8B, compare *lanes 1* and *3* and Figure 8C). Both these results corroborate the report by V. Ramesh (2001). In Figure 6, the $tRNA^{ser}$ control was more intense in *lane 1*, indicating that the $tRNA_i^{Met}$UAA was also uncharged.

Interestingly, $tRNA_i^{Met}$UGA does not show a charged band at the position of wild type $tRNA_i^{Met}$ (Figure 7B, compare *lanes 1* and *3*). However, a slower migrating band is seen and it can be deacylated (Figure 7B, compare *lanes 1* and *2*). This indicated the $tRNA_i^{Met}$UGA is being charged *in vivo*. Two possibilities can explain the slower migration: the tRNA is charged with a positive amino acid, which slows its migration towards the anode during the acid urea PAGE; or, it could be charged with a neutral amino acid and a post-transcriptional modification affects its migration rate. The second option would require that base treatment remove the modification to account for the single band in B*2*.

**Recombineering and the construction of mutant *bgaH* reporters**

*bgaH* is an archaeal β-galactosidase gene from *Haloferax lucentensis* (formerly *Haloferax alicantei,* Gutierrez *et al.* 2002). It was isolated from a mutant strain with increased activity, and cloned as a 5.4kb genomic fragment into the vector pMDS20 to produce pMLH32

(Holmes, 2000). In the same paper it was also shown that when the plasmid was transformed into *H. volcanii* WFD11, a strain with no detectable β-galactosidase activity, active β-galactosidase was produced and could be easily assayed for with ONPG or visualized with X-gal.

This reporter has since been used to study transcriptional promoters and Shine-Dalgarno sequences in archaea (Gregor and Pfeirer, 2005 & Sartorius-Neef and Pfeifer, 2004). Here I describe another application: its use in assaying translation initiation and elongation.

An outline for construction of the *bgaH* reporters with the mutant start codons UAG, UAA, UGA, and GUC is presented in Figure 9. The fidelity of standard site directed mutagenesis is reduced for plasmids greater than 8kb (Stratagene). Since the pMLH32 plasmid is approximately 13.5kb, the smaller *Hin*dIII/*Kpn*I *bgaH* fragment was cloned into pUC18 and mutagenesis was performed. To facilitate cloning, a two step strategy was used to introduce the modifications to the *bgaH* start codon. First, a unique *Pst*I site was introduced at the start codon (Figure 10A) and then screened for its presence (Figure 11A). Then, the site was replaced with the desired start codon (Figure 10B) and screened for the absence of the *Pst*I site (Figure 11B). The same strategy was used to create the leaderless mutants lacking the 5' untranslated region (UTR) of the *bgaH* mRNA (Figure 10 C and D). All pUC.bgaH mutants were then confirmed by DNA sequencing.

Cloning the mutant *Hin*dIII/*Kpn*I *bgaH* fragment back into the pMLH32 plasmid could not be done using the same restriction enzyme sites because there are two *Kpn*I sites in the pMLH32 plasmid, one in the original vector and the other in the *bgaH* gene (Figure 2). At first, an attempt was made to remove the second *Kpn*1 site of pMLH32. Briefly, partial digestion with *Kpn*I was carried out to obtain linearized plasmid with the 4 nucleotide overhangs of the restriction site. S1 nuclease treatment was then used to remove the overhangs and produce blunt ends. Finally, ligation was done and the plasmids were to be screened to ensure that only the correct *Kpn*I site was removed. A *Kpn*I digestion time was chosen such that the plasmid was only cut once and S1 nuclease treatment was optimized for plasmid concentration (data not shown). However, after ligation, there were no colonies and a new strategy was pursued.

Recombineering presented itself as a novel solution to the problem. It uses the λ-red genes to accomplish homologous recombination between DNA with small homologies (reviewed in Court, Sawitzke, and Thomason, 2002). Typically the technique has been used to modify large BACs and various genomes directly (Warming *et al.* 2005 and Datsenko and Wanner, 2000) and

so its application here was not immediately apparent. However, because the mutated *Hin*dIII/*Kpn*I *bgaH* fragments from pUC.bgaH are nearly entirely homologous with the *bgaH* gene in pMLH32, they can be used for recombination. The only requirement was creating some form of selection for recombinants, and that was accomplished by linearizing pMLH32 in the region to be combined (Figure 9D).

A unique restriction site near the start codon was initially used to linearize pMLH32, however, as distance from the restriction site (the gap) to the desired mutation increases it becomes more likely a crossover will occur between them. To make the selection as accurate as possible, the first recombineering step introduced a unique *Pst*I restriction site at the codon to be mutated. This made screening simple (Figure 12A), and the second round of recombineering was also easily screened for (Figure 12B). All plasmids were confirmed by sequencing and passaged through a *dam*$^-$ *E. coli* strain for transformation of *H. volcanii*.

Creating *bgaH* reporters for studying nonsense suppression used the same scheme. Mutagenesis was used on pUC.bgaH to change the serine at codon 184 to a *Pst*I site and subsequently to UAG and UAA. The *bgaH* fragment containing the *Pst*I site was then recombined with *Bcl*I linearized pMLH32. Because of the distance between the gap and the target site slightly more clones had to be screened (data not shown). In the second step, fragments with the mutated codons were used during recombineering to replace the *Pst*I site at the 184 position.

### *bgaH* as a reporter for studying translation initiation in *H. volcanii*

In the first series of double transformants of WFD11, ONPG assays indicated that *bgaH* activity was lost when cells entered late stationary phase, suggesting maintenance issues. A *radA* deficient volcanii strain, DS52, was obtained from the Dyall-Smith lab. This enzyme is related to the recA/RAD51 family and therefore the host lacks recombination and offers greater plasmid stability (Woods and Dyall-Smith, 1997). However, the paper also indicates this new strain is not compatible with the pHV2 replicon of the pWL vectors (tested with pWL102). Mutant tRNAs expressed from pWL201 are, therefore, unlikely to continue to be made, so care must be used to sample for *bgaH* activity at shorter time points (before stationary phase).

To quickly test the double transformants, the ONPG assay for β-galactosidase was performed as described (Holmes, *et al.* 1997). However, work in this laboratory had previously

used the Beta-Glo reagent (Promega) for precise quantification of *LacZ* β-galactosidase activity (Koehrer, Sullivan, Rajbhandary 2004). The Beta-Glo reagent buffer was unknown, but in low salt buffers *BgaH* loses activity within minutes. It wasn't known if the two activities would be compatible, and so different reaction conditions were tested: The buffer supplied with the Beta-Glo reagent, a high salt buffer, and the supplied buffer with 20% sorbitol. Sorbitol was tested because during the biochemical isolation of *bgaH* it was shown to stabilize the enzyme without the need for high salt. It also only minimally interferes with the ONPG assay (Holmes, *et al.* 1997).

WFD11 was transformed with pMLH32, extracts were made, and the results of assay with the Beta-Glo reagent in different buffers are presented in Table 1. Adding sorbitol should have had a minimal effect, or, if there were salt instability problems, it should have increased the activity. The results showed it actually decreased the β-galactosidase activity indicating it was incompatible with the Beta-Glo reagent and unnecessary. Increasing the salt concentration also gave lower levels of activity, indicating the enzyme was active in the stock buffer.

Triton X-100 lysis step used only 11% as many cells, and when that was taken into account the lysis step significantly improved the assay. However, in that first experiment Difco Bacto-Peptone was being used in the media, which had been shown to cause lysis (Kamekura, *et al.* 1988). This means the non-lysed cells might have been partially lysed and so their values were likely higher than they should be. The data in Table 1 show that the Beta-Glo reagent is compatible with the *H. volcanii* system, that lysis should first be performed on the cells, and that the stock Beta-Glo buffer should be used.

The second experiment also confirmed the need for a lysis step. Oxoid bacto-peptone was used in the media and the effects of lysis are even more intense. In this experiment the previously created mutant *bgaH* initiation codons were tested for their activity. As expected, only the wild-type *bgaH* reading frame produced β-galactosidase levels that were significantly above background (Table 2). When the pMLH32 based mutant *bgaH* genes were co-expressed with their complementary tRNAs, the uncharged tRNAs (UAA and UAG) did not have any activity. The charged $tRNA_i^{Met}GUC$ could initiate translation from the GUC start codon in pMLH32M1GUC. This agrees with prior work done in this laboratory with the archaeon *Halobacterium salinarum* (Srinivasan, Krebs, and RajBhandary, 2006). Interestingly, the charged $tRNA_i^{Met}UGA$ did not initiate translation from the mutant *bgaH* gene with UGA as the

initiation codon. Some possible explanations for this are: only certain codons can be used to initiate translation; the amino acid the tRNA is charged with is important, possibly for a/eIF2 binding (Drabkin and RajBhandary UL, 1998 and Yatime, Schmitt, Blanquet, and Mechulam, 2004); if the UGA is post-transcriptionally modified, that could be interfering; or the protein is being made, but the nature of the initiating amino acid destabilizes the protein or results in inactive protein.

In the third Beta-Glo experiment, the cells were assayed at earlier stages of growth, all within 0.3-0.8 $OD_{600}$. The $tRNA_i^{Met}GUC$ increased *bgaH* level more than 10 fold over the mutant start codon reporter alone (Table T3). However, when $OD_{600}$ was used as a standard the values no longer made sense. This indicates that total protein will have to be used as the standard when computing relative values, and that harvesting at nearly identical $OD_{600}$ is important. The samples in this table were assayed twice and measured in duplicates, with a maximum standard deviation of 5.1%.

**Future work: Translation of *bgaH* with leaderless transcripts**

In contrast to bacteria, the archaea have a large proportion of mRNA transcripts that are leaderless, that is they contain no or only a few nucleotides in their 5' UTR (Torarinsson, Klenk, & Garrett, 2005). It is unknown if the leadered and leaderless transcripts will behave differently when assayed for translation initiation. However, it has been shown that a leaderless version of *bgaH* increases its activity (Sartorius-Neef & Pfeifer, 2004).

Leaderless reporters have been made in which the AUG start codon is mutated to UAG, UAA, UGA, and GUC. Since only the UGA and GUC decoding tRNAs are charged, co-expression studies need only use those tRNAs and their respective reporters. The main purpose of this experiment will be to determine if the charged initiator derived tRNAs have different activities in initiation on leaderless mRNA versus leadered mRNA. Also, since little is known about translation initiation in archaea, there is a possibility that the UGA decoding tRNA could be active with a leaderless mRNA construct.

**Future work: Adapting this system to study nonsense suppression**

In the archaea, there is one known example of natural nonsense suppression (Srinivasan, James, and Krzycki, 2002). There are no published examples of using nonsense suppression with introduced tRNAs.

Using the already presented cloning/recombineering strategy, the *bgaH* reading frame was modified at codon 184, changing a serine codon to UAA and UAG. At the same time, $tRNA_3^{Ser}$ derived tRNAs that could decode UAA and UAG were generated and cloned into the pWL201 expression vector. Serine tRNA was chosen because seryl-tRNA synthetase does not use the anticodon sequence as an identity element and should, therefore, have no problem in charging the mutant $tRNA_3^{Ser}$ (Asahara H, *et al.*, 1994).

A mistake during cloning led to the loss of 7 nucleotides immediately 3' of the tRNA. It is unknown if this sequence is required for proper processing of the tRNA, however, the mutants can still be tested regardless. Once the tRNAs are shown to be charged, their coexpression with the appropriate reporters will demonstrate whether nonsense suppression is possible in the archaea.

If it does work, the specificity of nonsense suppression can also be determined as in Köhrer, Sullivan, and RajBhandary (2004). Briefly, in mammalian cells it was shown that the UAG and UAA decoding suppressor tRNAs were specific for their cognate codons. In contrast, in *E. coli* and in bacteria in general, it is known that UAA decoding tRNA can also suppress a UAG nonsense codon.

**Future work: Identifying tRNA identity elements**

Various proteins recognize tRNAs through the use of identity elements, nucleotides usually located in the acceptor stem and anticodon stem and loop. The identity elements of the initiator tRNA are unknown for archaea, although they have been studied for the other two domains (Stortchevoi, Varshney, and RajBhandary, 2003, also presented Figure 3). The reporter system developed here can be readily used to study this aspect of translation initiation.

The aminoacylated GUC decoding $tRNA_i^{Met}$ will be mutagenized at putative identity elements. Then, when assayed with the *bgaH* reporter any changes in activity will indicate the importance of that nucleotide. For example, one study will change the anticodon stem of the methionine initiator tRNA to that of the methionine elongator tRNA. Since this region is

believed to help direct the tRNA to the ribosomal P site, the new tRNA should not have any activity when assayed. Bacterial initiator tRNAs require the 1-72 base pair to be mismatched, whereas eukaryotic initiator tRNAs require the A1:U72 base pair (Farruggio, Chaudhuri, Maitra, and RajBhandary, 1996). As the archaeal tRNA also has A1:U72 base pair, it will be mutated to a G1:C72 base pair and an A1:C72 mismatch. If the $tRNA_3^{Ser}$ derived suppressor tRNAs are found to be active this strategy can also be used to study the identity elements that prevent an initiator tRNA from acting in elongation in archaea.

**Materials and Methods:**

**Strains and Plasmids**

*See Attached Table*

**Media and Reagents**

*See Attached Table*

**Transformation of *E. coli***

Standard procedures were used to grow *E. coli* (Sambrook, 1989). Competent cells were generated as described by Inoue *et al.* (1990).

**Purification of plasmids from *E. coli***

QIAprep Spin Miniprep Kits (Qiagen) were used for isolation of plasmid DNA from *E. coli*. Plasmid from 3 ml of overnight culture was isolated according to the manufacturer's instructions. Washing with Buffer PB was included when the *endA*$^+$ strain GM2163 was used. When isolating the larger pMLH32 and pWL201 based plasmids, elution buffer was preheated to 75°, added to the spincolumns and incubated for 5 min at 42°, and then the DNA was eluted by centrifugation.

For large scale preparations of *dam*$^-$ DNA from GM2163 and ER2925 the QIAFilter Midi and Maxi kits were used (Qiagen). However, for unknown reasons the yields were quite low. For this reason the plasmids were isolated as minipreps and pooled.

**Site directed mutagenesis**

QuikChange site directed mutagenesis was done according to the manufacturers instructions (Strategene). In addition, *PfuTurbo* DNA polymerase (Stratagene) was used for PCR, with extension times of 1 min per KB.

When using synthesized oligonucleotides to create full length tRNA genes, 10 picomoles of each 90nt oligonucleotide was used as a template and combined with 10 picomoles of each 21nt primer. Also, all mutagenesis involving tRNA genes used 5% DMSO in the reaction.

**Cloning of the *H. volcanii* mutant initiator tRNA genes**

The initial work to generate the tRNA expression plasmids was done by V. Ramesh (Ramesh and RajBhandary, 2001). Briefly, PCR was used to amplify the $tRNA_i^{met}$ gene from isolated *H. volcanii* genomic DNA. Restriction sites (*Xba*I and *Bam*HI) were introduced during PCR and the gene was cloned into the tRNA expression cassette of pUCsptProM to create pUCsptHvMet$_i$. That plasmid was then used as a template for Quik Change mutagenesis to generate the anticodon mutants that could decode UAG and GUC (U35A36 and G34C36 mutants respectively). Finally, the expression cassette was digested with *Hin*dIII and *Eco*RI, the fragment isolated, and cloned into the pWL201 vector.

For this work, pUCsptHvMet$_i$ was used as a template for site mutagenesis, done in the presence of 5% DMSO. The primers used were as follows:

Mutagenesis to pUCsptHvMetiUAA (U34U35A36)

| | |
|---|---|
| HvMetiUAA | TTCCGCCGGGCTttaAACCCGGAGATC |
| HvMetiUAAR | GATCTCCGGGTTtaaAGCCCGGCGGAA |

Mutagenesis to pUCsptHvMetiUGA (U34C35A36)

| | |
|---|---|
| HvMetiUGA | TTCCGCCGGGCTtcaAACCCGGAGATC |
| HvMetiUGAR | GATCTCCGGGTTtgaAGCCCGGCGGAA |

The cloning site in pWL201 had not been sequenced and was only described as *Hin*dIII/*Cla*I/*Eco*RI. To facilitate the future cloning of a synthetase, all the tRNA plasmids had their second *Sst*I site mutagenized to *Cla*I (Figure 2A). This would allow the tRNA gene to be cloned into pWL201 using the *Hin*dIII/*Cla*I sites and then the synthetase could be cloned using the *Cla*I/*Eco*RI sites. The primers used were as follows:

| | |
|---|---|
| pUCspt-Sst2Cla | TAAAGTAGCAGT**atcgatGAATTC**ACTGGC |
| pUCspt-Sst2ClaR | GCCAGT**GAATTCatcgat**ACTGCTACTTTA |

However, after creating the tRNA anticodon mutants with the *Cla*I site, they could not be successfully cloned into pWL201. This led to an investigation of the actual sequence of the cloning site. A complementary primer to HvMet$_i$ was used to sequence into the upstream region of pWL201HvMet$_i$. Using that data, primer pWL_ES1 was designed to read the cloning site in pWL201. The primers and MCS are listed below (with the *Hin*dIII *Cla*I and *Eco*RI restriction sites in bold)

| | |
|---|---|
| HvMetiR | GGTTATGAGCCCGGCGGAATCT |
| pWL_ES1 | CCAACTACTCGAATCGGGCG |

pWL201 Cloning Site ...AAAG**AAGCTTATCGAT**GATAAGCTGTCAAACATGA**GAATTC**TTGA

The adjacency of the *Hin*dIII and *Cla*I sites prevented this cloning strategy from working, and so the original *Hin*dIII and *Eco*RI sites were used. However, when making the mutants pUCsptHvMet$_i$UAA and pUCsptHvMet$_i$UGA, the *Sst*I to *Cla*I mutation was done first, and so the final pWL201 vectors retain the *Cla*I site, although it is not useful for cloning as it is adjacent to the *Eco*RI site.

After confirming their sequences, the completed vectors were passaged through a *dam*$^-$ strain, GM2163 or ER2925.

**Cloning of the *H. volcanii* ser-3 suppressor tRNA genes**

The full $tRNA_3^{Ser}$ sequence was published by R. Gupta (1986) as accession# M35748. It was synthesized as two 90nt complementary oligonucleotides that extended 6 nucleotides past the *Xba*I and *Bam*HI sites used during the cloning of pUCsptHvMeti. These oligonucleotides were combined with two complementary 21nt primers and subjected to PCR amplification. Sequences are as follows:

| | |
|---|---|
| Hv Ser-tRNA-3 | gccaggatgg ccgagcggta aggcgcacgc<br>ctGGAaagcg tgttccctct gggatcgggg<br>gttcaaatcc ctctcctggc g (cca) |

| | |
|---|---|
| HvSer3 UAA-PCRF | GGGGAC**TCTAGA**CTGTTGTTGATTCgccaggatggccgagcggtaaggcgcacgccttTAaagcgtgt<br>tccctctgggatcgggggttca |
| HvSer3 UAA-PCRR | CCCGG**GGATCC**GGAGTTGAGGTCGGcgccaggagagggatttgaacccccgatcccagagggaacacg<br>cttTAAaggcgtgcgccttacc |

| | |
|---|---|
| HvtRNA-PCRF | GGGGAC**TCTAGA**CTGTTGTTG |
| HvtRNA-PCRR | CCCGG**GGATCC**GGAGTTGAGG |

Note that the lowercase nucleotides indicate the serine tRNA, whereas uppercase nucleotides are complementary to pUCsptHvMeti. This sequence produced a modified tRNA

with a UUA anticodon, hence the UAA decoding tRNA. The PCR product was digested with *Xba*I and *Bam*HI, isolated on an agarose gel, and cloned into the pUCspt cassette.

The $tRNA_3^{ser}UAA$ vector, pUCsptHvSerUAA served as a template for the UAG decoding tRNA. Standarard mutagenesis was done with the following primers:

| | |
|---|---|
| HvSer3UAG | aggcgcacgcctCTAaagcgtgttccc |
| HvSer3UAGR | gggaacacgcttTAGaggcgtgcgcct |

It is important to note that a mistake was made while cloning. The 7 nucleotide sequence, TGGTTTG, should have immediately followed the tRNA, preceding the CCGACC sequence. It is unknown if this sequence will affect post-transcriptional processing. This mistake was not noticed until after the tRNAs had been cloned into the pWL201 vector and so they will still be tested for functionality.

**Modifying identity elements in the *H. volcanii* initiator tRNA genes**

Since the GUC decoding initiator tRNA is active in translation initiation, it was further modified to study initiator tRNA identity elements. As multiple mutations were being made simultaneously, it was easier to follow the synthesis strategy used with the serine tRNAs and order the entire tRNA as two oligonucleotides for use in PCR. HvtRNA-PCRF&R were used for amplification with the following:

| | |
|---|---|
| Hv met-tRNA-i | agcgggatgg gataGccagg agattccgcc<br>gggctCATaa cccggagatc ggtagttcAa<br>atctacctcc cgcta (cca) |

| | |
|---|---|
| HvMetACSL | GGGGAC**TCTAGA**CTGTTGTTGATTCagcgggatgggatagccaggagattccgccgCActGACaa<br>TGcggagatcggtagttcaaatcta |
| HvMetACSLR | CCCGG**GGATCC**GGAGTTGAGGTCGGtagcgggaggtagatttgaactaccgatctccgCAttGTC<br>agTGcggcggaatctcctggctatc |

The full $tRNA_i^{met}$ sequence was published by R. Gupta (1984) as accession# K00307. However, when that sequence was used as an input to blast the *H. volcanii* contig sequences (Compared online at http://halo.umbi.umd.edu/cgi-bin/blast/blast_hvo.pl), the G at position 15 and A at position 59 differed from the original sequence published by R. Gupta. This indicated a mistake in his results, as the pUCsptHvMeti clones lifted from genomic DNA also contained

these substitutions. The ordered oligonucleotides and the clones created herein do not contain this error.

The sequence presented above not only created the GAC anticodon, but changed the anticodon stem loop to match that of the elongator methionine tRNA (R. Gupta, 1984). Again, the accidental loss of 7 nucleotide occurred during ordering of the sequences.

Two point mutations of $tRNA_i^{Met}GUC$ were planned, changing the A1:U72 pair to the mismatch A-C and subsequently the strong base pair, G-C.

| | |
|---|---|
| HvMetGUCA1G | TGTTGTTGATTCGgcgggatgggat |
| HvMetGUCA1GR | atcccatcccgcCGAATCAACAACA |

| | |
|---|---|
| HvMetGUCT72C | tctacctcccgcCaCCGACCTCAACT |
| HvMetGUCT72CR | AGTTGAGGTCGGtGgcgggaggtaga |

None of the modified clones have been transferred to pWL201. As the 7nt sequence may be important, the T72C oligonucleotides will need to be reordered.

**Transformation of *H. volcanii***

The protocol used was adapted from that presented online in the Halohandbook (Dyall-Smith, 2006). WFD11 was streaked for single colonies onto (18%) modified growth medium (MGM) plates. A single colony was used to inoculate 3 ml of MGM and incubated at 37° until late log phase. Growth times at this stage varied greatly, and could range from overnight to several days. The culture was then diluted $1/10^{th}$ and used to inoculate 25 ml of MGM and grown 1-2 days at 37° until late log phase ($A_{600}$ 0.8-1.0).

Cultures were transferred to 50 ml Falcon tubes and centrifuged 15 min at 5500g. Pellets were resuspended in 5 ml Buffered Spheroplasting Solution, and again centrifuged 15 min at 5500g. The Halohandbook protocol called for lower speeds and times, but this did not yield stable pellets. Finally, the cells were resuspended in 2.5 ml Buffered Spheroplasting Solution. Cells at this point should have been able to be quick frozen, stored at -80°, and then used. However, no such attempt yielded transformants and so all cells were prepared and used fresh.

100 µl of 0.5M EDTA (pH 8.0) was gently mixed with 1 ml of the concentrated cells and allowed to incubate 10 min at 37° to form spheroplasts. Meanwhile, 1 µg of unmethylated DNA

(~3 µl of a Qiaprep miniprep) was placed in a 1.5 ml tube. After the incubation, 100 µl of the spheroplast cells were added to the DNA, mixed gently, and incubated 5 min at room temperature. Next, an equal volume (100 µl) of 60%$PEG_{600}$ was added and mixed. This step often failed, producing a viscous solution that would not yield transformants and so was sometimes repeated. The cells were then incubated 30 min at room temperature.

To plate the cells the PEG had to be removed. This was accomplished by adding 1 ml MGM to the 1.5 ml tube, centrifuging 5 min at 6500, and then resuspending in another 1 ml MGM. Outgrowth was then done for 2 hr at 37° in the same tubes, followed by a final centrifugation to concentrate the cells into ~100 µl. That entire volume was then used to plate onto selective media directly. The plates were placed in Ziplock bags and incubated at 42° for ~7 days.

Alternatively, a procedure more similar to the original method of Cline (Cline, *et al.* 1989) was also used, but the extra precautions against lysis were found to be unnecessary when working with *H. volcanii.*

Cells were diluted 1/25$^{th}$ into 50 ml of *H. volcanii* Growth Medium and harvested at an $A_{600}$ of 1.0 (~25 hr at 37°). They were then resuspended in 5 ml Spheroplasting Buffer. To each 200 µl of suspension 20 µl 0.5M EDTA (pH 8.0) was added. ~1 µg of plasmid in 20 µl of Speroplasting Buffer was added and mixed, followed by 240 µl of 60% PEG Buffer. This was allowed to incubate for 20 min, after which it was diluted with 9 ml Spheroplasting Dilution Buffer. Cells were then centrifuged and resuspended in 1 ml of a 1:1 mixture of Spheroplast Dilution buffer and *H. volcanii* Growth Medium. A recovery period of 12 hr at 37° was followed by plating dilutions onto *H volcanii* Solid Medium. The plates were then placed inside Ziplock bags and incubated at 37° for ~7 days.

Mevinolin selection was done at 4 mg/liter (prepared as the sodium salt, Kita T, *et al.* 1980). Novobiocin selection was done at 0.3 mg/liter. Oxoid Bacto-Peptone, was used for media.

**Isolation of total charged tRNA (under acidic conditions from *H. volcanii*)**

A single colony was used to inoculate 3 ml of *H. volcanii* growth medium and incubated at 37° for 3 days. All subsequent steps are done on ice. The cells were pelleted and resuspended

in 0.3 ml of 0.3 M NaOAc (pH 4.5) / 10 mM EDTA. Two extractions were performed with an equal volume of equilibrated phenol (vortexed for 5 seconds, placed on ice/water for 5 min, vortexed again, then centrifuged). The aqueous phase was then precipitated with 2.5 volumes of ethanol. The precipitate was washed with 70% ethanol and then dissolved and stored in 20 µl 10 mM NaOAc (pH 4.5) / 1 mM EDTA.

**Acid Urea PAGE and Northern Blotting**

Acid urea polyacrylamide gel electrophoresis (PAGE) and Northern Blotting were performed as described by Varshney *et al.* (1991). Modifications were as follows:

tRNA was isolated as above, and samples were prepared as either 0.1 or $0.2A_{260}$ units of tRNA. Uncharged controls were made by increasing the sample buffer to 0.5 M Tris-Cl (pH 9.5), and incubating at 37° for 45 min. tRNA samples were then mixed with an equal volume of 2x Sample Buffer.

The gel was cast as a 6.5% acid urea PAGE and covered with an aluminum sheet to aid in heat distribution. The apparatus was placed in the 4° cold room and prerun at 400v for 30 min. Samples were then added, with each well being cleared of urea immediately before loading. The gel was then run at 500v until the Bromophenol Blue was just running off the bottom of the gel (~16 hr, no more than 20 hr).

The gel was then excised between the Bromophenol Blue and Xylene Cyanol and originally blotted onto Hybond-$N^+$ (Amersham). However, Nytran SPC (Whatman) was found to bind more tRNA, requiring shorter exposure times and was, therefore, preferred. The acrylamide/membrane cassette was then placed in Transfer Buffer and moved to the 4° cold room. The transfer was prerun at 10v for 20 min then 40v for 2 hr. The acrylamide was then removed and crosslinking was done by drying at 75°C for 3 hr.

The membrane was pre-hybridized 2-6 hr at 42°C in 6xSSC, 10x Denhardt's solution, and 0.5% SDS. Importantly, this solution was filtered through a 0.45 µM filter before being used to block the membrane. Hybridization was done overnight at 42°C in 6xSSC, 0.1% SDS, and 0.25 nM of $^{32}P$ labeled probes (all filtered). The membrane was then washed twice at RT for 10 min with 1-6xSSC. Probes were labeled with T4-PNK (NEB) in the presence of 3000 Ci/mmol $\gamma$-$^{32}P$-ATP. Visualization was performed by exposing to Kodak Biomax XAR film.

The probes used for labeling are listed below. The first 3 did not target the anticodon, whereas the latter 5 only target a specific anticodon. The final probe targeted serine and was used as an internal control.

| | |
|---|---|
| HvMetiP1R | atttgaactaccgatctccgggtt |
| HvMetiP2R | ggtagcgggaggtagatttgaa |
| HvMetiP3R | ctcctggctatcccatcccgct |
| | |
| HvMetiP-AUG | atctccgggttATGagccc |
| HvMetiP-UAG | atctccgggttTAGagccc |
| HvMetiP-UAA | atctccgggttTAAagccc |
| HvMetiP-UGA | atctccgggttTGAagccc |
| HvMetiP-GUC | atctccgggttGTCagccc |
| | |
| HvSerP | cagagggaacacgctttcc |

**Recombineering**

The following protocol is an adaptation of one provided by Soren Warming (Warming *et al.* 2005). SW102 cells were used unless mentioned otherwise.

Cultures (3 ml) were inoculated from a single colony and grown overnight in $LB_{tet}$ at 30°. Cells were diluted $1/50^{th}$ into 50 ml $LB_{tet}$ and grown in a 32° shaking water bath. After 3 hr the cells were removed at an $OD_{600}$ ~0.4-0.6. If heat induction was required, the cells were incubated in a 42° shaking water bath for 15 min. Induced and uninduced cells were cooled and split into 10 ml portions (in 15 ml Falcon tubes).

The cells were pelleted for 5 min at 5000g. The supernatant was removed and the excess media blotted onto a towel. The pellet was then resuspended in 1 ml of ice cold $H_2O$ by shaking in an ice/water slurry. This step required patience, but vortexing should not be used. The volume of cold $H_2O$ was brought to 10 ml, inverted a few times, and centrifuged again. A second washing with $H_2O$ was performed (two total), and the pellet was allowed to dry slightly on a paper towel. ~80 µl $H_2O$ could not be removed and it was used to resuspend the pellet. If the volume was higher or lower, it was brought to 80 µl and placed on ice.

40 µl of cells were mixed with < 5 µl DNA and electroporated in a 1mm cuvette at 1.8kV/cm. 1 ml of LB was immediately added and then the mixture was transferred to a 5 ml polypropylene tube for outgrowth in a 32° shaking water bath. For genomic changes, the outgrowth time was 4 hr, for plasmids it was reduced to 1 hour. For selections on minimal media,

pellets were washed 2x in 1 ml of M9 medium. Dilutions were spread on appropriate media and incubated at 30°.

**Cloning the *bgaH* initiation codon mutants**

Due to the large size of the pMLH32 vector (13.5kb), standard PCR mutagenesis could not be used and so a multipart strategy was employed. Essentially, a fragment of the gene was subcloned into pUC where mutagenesis was performed. The same restriction sites could not be used to transfer the fragment back into the pMLH32 vector though. Recombineering was therefore used, which selected only pMLH32 plasmids which recombined with the mutated pUC fragment (Figure 9). The restriction site for *Pst*I was used to simplify screening, as it was unique in both constructs and allowed mutants to be identified by different migration on an agarose gel (Figures 11 and 12).

pMLH32 was completely digested with *Hin*dIII/*Kpn*I. The fragments were separated on an agarose gel, and the ~1.5KB fragment containing the *bgaH* start codon was eluted with a Gel Extraction Kit (Qiagen). The insert was then ligated into pUC18 which had also been digested with *Hin*dIII/*Kpn*I (Figure 9A). This cloning was done in XL1 blue and the transformants were selected on $LB_{amp}$. The insert was verified by DNA sequencing of the first several hundred nucleotides, creating pUC.bgaH.

Standard PCR based mutagenesis was done to add a unique *Pst*I site to the pUC.bgaH vector, replacing the ATG start codon with CTGCAG (Figure 10A). The sequence is presented below, with the ATG indicating the start site, and the underlined regions being where the primers are targeted.

...gttgatcattgtgtATGacagttggtgtctg... (Target)
...gttgatcattgtgt**CTGCAG**acagttggtgtctg... (Product)

| | |
|---|---|
| bgaH1pstI | GTTGATCATTGTGT**ctgcag**ACAGTTGGTGTCTG |
| bgaH1pstIR | CAGACACCAACTGT**ctgcag**ACACAATGATCAAC |

Since the *Pst*I site was unique, screening for positive clones consisted of digesting plasmid minipreps with *Pst*I and comparing their mobility to that of the parent clone. The single restriction site linearized the positive clones and their mobility was retarded on an agarose gel, creating pUC.bgaHM1pstI (Figure 11A).

The *Pst*I site allowed for more rapid screening of subsequent mutants. The site was mutagenized with primers designed to change the start codon to a UAG, UAA, UGA, or GUC (Figure 10B). Normally these small nucleotide mutations could not be easily screened for, but, the loss of the *Pst*I site meant that any restriction digest with that enzyme would fail to linearize the plasmid, and hence the super coiled (ie positive) plasmids would run faster than the linearlized parental plasmid DNA on an agarose gel (Figure 11B). The primers are listed below, and were used to create pUC.bgaHM1UAG, pUC.bgaHM1UAA, pUC.bgaHM1UGA, and pUC.bgaHM1GUC.

gttgatcattgtgt**CTGCAG**acagttggtgtctg... (Target)
...gttgatcattgtgtUAGacagttggtgtctg... (Product)

| | |
|---|---|
| bgaH1TAG | GTTGATCATTGTGTtagACAGTTGGTGTCTG |
| bgaH1TAGR | CAGACACCAACTGTctaACACAATGATCAAC |
| | |
| bgaH1TAA | GTTGATCATTGTGTtaaACAGTTGGTGTCTG |
| bgaH1TAAR | CAGACACCAACTGTttaACACAATGATCAAC |
| | |
| bgaH1TGA | GTTGATCATTGTGTtgaACAGTTGGTGTCTG |
| bgaH1TGAR | CAGACACCAACTGTtcaACACAATGATCAAC |
| | |
| bgaH1GUC | GTTGATCATTGTGTgtcACAGTTGGTGTCTG |
| bgaH1GUCR | CAGACACCAACTGTgacACACAATGATCAAC |

The pMLH32 vector DNA isolated from *dam*$^-$ cells was digested overnight with *Bcl*I (the enzyme is blocked by *dam* methylation). This linearized the vector and it could no longer transform cells even under recombineering conditions. To create a circularized vector the backbone would have to be recombined with something to bridge the gap. The mutagenized pUC.bgaHM1pstI plasmid provided that in the form of the isolated *Hin*dIII/*Kpn*I fragment (Figure 9D). Since the *Bcl*I site was only 9 nucleotides away from the *bgaH* start codon, nearly all the clones carried the desired mutation. However, as the distance between the restriction site and the desired mutation increases, the chances of a crossover event occurring between them rises and the likelihood of finding clones deceases.

Since the pMLH32 vector was so large, supercoiled and linearlized vector migrate equivalently on an agarose gel. Therefore screening was accomplished by using *Kpn*I and *Pst*I, with the appearance of an extra band indicating a positive clone (Figure F8, part A).

This newly screenable vector, pMLH32M1pstI, was then recombined with the pUC.bgaH start codon mutants and desired clones were those which lost the *Pst*I site (Figure 12B). All clones were verified by DNA sequencing with the following primer and passaged through a dam$^-$ *E. coli* strain (GM2163 or ER2925) before being used for transformation of *H. volcanii*.

| | |
|---|---|
| pML_ES2 | GCGACCGGGTCTCGCGTTCG |

**Cloning the leaderless *bgaH* mutants:**

Primer extension performed on the mRNA transcript indicated that transcription start site was the A at -34 relative to the AUG (Holmes and Dyall-Smith, 2000). The sequence is presented below, with the ATG indicating the start site, the underlined regions are where primers are targeted, and the italicized region is the 5' UTR.

…ggatatcaatcggtgctcagacaccg*gaaagaactatatctcaccacgttgatcattgtgt*ATGacagttggtgtctg… (wt)
…ggatatcaatcggtgctcagacaccg*gaaagaactatatctcaccacgttgatcattgtgt***CTGCAG**acagttggtgtctg… (target)
…ggatatcaatcggtgctcagacaccg*GTAG*acagttggtgtctg… (product)

The mutagenesis strategy was identical to the one presented above and used pUC.bgaHM1pstI as a (target) template. Since the *Pst*I site would be removed along with the 5' UTR, the screening strategy was identical and the clones yielded mutants with shorter leader regions (data not shown). Note that all the mutants retain the required purine as the transcription start site (Palmer & Daniels, 1995). However, the UGA clone was slightly more difficult. As archaea can use AUG as well as GUG and UUG to initiate translation (Torarinsson, Klenk, & Garrett, 2005), the shortest UGA containing sequence without an initiation codon is GCUGA or ACUGA. The former was chosen and so all the clones used guanine as the starting transcription nucleotide. The created plasmids were named pUC.bgaHM1UAGL, pUC.bgaHM1UAAL, pUC.bgaHM1UGAL, pUC.bgaHM1GUCL and pUC.bgaHM1AUGL.

| | |
|---|---|
| bgaH1TAG-L | GTGCTCAGACACCGGgtagACAGTTGGTGTCTG |
| bgaH1TAG-LR | CAGACACCAACTGTctacCCGGTGTCTGAGCAC |
| | |
| bgaH1TAA-L | GTGCTCAGACACCGGgtaaACAGTTGGTGTCTG |
| bgaH1TAA-LR | CAGACACCAACTGTttacCCGGTGTCTGAGCAC |
| | |
| bgaH1TGA2-L | GTGCTCAGACACCGGgctgaACAGTTGGTGTCTG |
| bgaH1TGA2-LR | CAGACACCAACTGTtcagcCCGGTGTCTGAGCAC |

| | |
|---|---|
| bgaH1GUC-L | GTGCTCAGACACCGGgtcACAGTTGGTGTCTG |
| bgaH1GUC-LR | CAGACACCAACTGTgaccCCGGTGTCTGAGCAC |
| | |
| bgaH1AUG-L | GTGCTCAGACACCGGgatgACAGTTGGTGTCTG |
| bgaH1AUG-LR | CAGACACCAACTGTcatcCCGGTGTCTGAGCAC |

**Cloning the *bgaH* nonsense codon mutants:**

To create nonsense codons in the *bgaH* open reading frame, the already described strategy was used (Figure 9). Briefly, mutagenesis was done on the desired codon, serine 184, to introduce a unique *Pst*I site to pUC.bgaH. That site was used for screening and was then mutated into the desired codons, UAA and UAG with the following primers:

| | |
|---|---|
| bgaH184pstI | GAACGACGTTTTGG**ctgcag**CAGCAGTACGACG |
| bgaH184pstIR | CGTCGTACTGCTG**ctgcag**CCAAAACGTCGTTC |
| | |
| bgaH184TAA | GAACGACGTTTTGGtaaCAGCAGTACGACG |
| bgaH184TAAR | CGTCGTACTGCTGttaCCAAAACGTCGTTC |
| | |
| bgaH184TAA | GAACGACGTTTTGGtagCAGCAGTACGACG |
| bgaH184TAAR | CGTCGTACTGCTGctaCCAAAACGTCGTTC |

Recombineering was then used to move the *Pst*I site at codon 184 into the *bgaH* reading frame of pMLH32. Finally, the desired mutants were introduced through recombineering as in part E. The clones were DNA sequenced with the following primer:

| | |
|---|---|
| pML_ES3_184 | CGGCTGTCACGAGACGG |

**Assay for β-Galactosidase: use of ONPG as a substrate**

This method is described by Holmes, *et al.* (1997). At room temperature, 700 µl *bgaH* buffer, 100 µl test cells, and 100 µl of 2% Triton X-100 in water were added to a plastic cuvette, covered with parafilm, and then vortexed for 10 seconds, with the detergent causing lysis. The reaction was started by adding 100 µl of an 8 mg/ml ONPG/BgaH Buffer solution and votexing for 3 seconds. The release of *o*-nitrophenol was followed spectrophotometrically by measuring the change in absorbance at 405nm. Samples were standardized against their $OD_{600}$. Here it was

only used as a qualitative assay, therefore, none of the samples were standardized to protein concentration.

**Assay for β-Galactosidase: use of Beta-Glo reagent**

The Beta-Glo reaction is adapted from the manufacturer's instructions (Promega). Reagents are stored at -20° and equilibrated to room temperature before each assay. Preparation of the reagent usually involves dilution of the substrate with 10 ml of the supplied buffer. Because the composition of the buffer is unpublished, a low salt and high salt reagent/buffer solution were also used in the assay. To make them, the reagent was first made as a 10x solution with 1 ml of the supplied buffer, and then brought to 1x with a modified buffer: the low salt dilution buffer was 20% sorbitol (w/v) in the supplied buffer (final 18% sorbitol); the high salt dilution buffer was *BgaH* buffer (final concentration 2.25 M NaCl).

Additionally, a lysis step was done identically to the one in the ONPG assay. 50 µl of the lysed cells were mixed with 50 µl of reagent in a 1.5 ml tube and thoroughly mixed by inversion. The samples were allowed to incubate at room temperature for 30 min and were then assayed in a SIRIUS luminometer (Berthold Detection Systems), with an integration time of 1 second. A pMLH32 transformed strain was assayed to generate a background which was subtracted from all measurements. If samples needed dilution, these cells were also used so that total cellular material stayed approximately constant.

## References:


Asahara H, Himeno H, Tamura K, Nameki N, Hasegawa T, and Shimizu M (1994) *Escherichia coli* Seryl-tRNA Synthetase Recognizes $tRNA^{Ser}$ by Its Characteristics Tertiary Structure. *J. Mol. Biol.* **236:** 738-748.

Cline SW, Lam WL, Charlebois RL, Schalkwyk LC, Doolittle WF (1989). Transformation methods for halophilic archaebacteria. *Can. J. microbial.* **35**: 148-152.

Coulson RM, Touboul N, Ouzounis CA (2007) Lineage-specific partitions in archaeal transcription. *Archaea.* **2**:117-125.

Court DL, Sawitzke JA, Thomason LC (2002) Genetic engineering using homologous recombination. *Annu. Rev. Genet.* **36**: 361-388.

Datsenko KA, and Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *PNAS.* **97**: 6640-6645.

Drabkin HJ and RajBhandary UL (1998) Initiation of protein synthesis in mammalian cells with codons other than AUG and amino acids other than methionine. *Mol Cell Biol.* **18**: 5140-5147.

Dyall-Smith, ML (2006). *The Halohandbook: Protocols for Halobacterial Genetics.* **V6**: http://www.microbiol.unimelb.edu.au/people/dyallsmith/HaloHandbook.

Farruggio D, Chaudhuri J, Maitra U, RajBhandary UL (1996) The A1 x U72 base pair conserved in eukaryotic initiator tRNAs is important specifically for binding to the eukaryotic translation initiation factor eIF2. *Mol Cell Biol.* **16**: 4248-4256.

Gregor D, and Pfeifer F (2005) In vivo analyses of constitutive and regulated promoters in halophilic archaea. *Microbiology* **106**: 1289-1238.

Grill S, Gualerzi CO, Londei P, Bläsi U (2000) Selective stimulation of translation of leaderless mRNA by initiation factor 2: evolutionary implications for translation. *EMBO J.* **19**: 4101-4110.

Gupta R (1984) Halobacterium volcanii tRNAs. Identification of 41 tRNAs covering all amino acids, and the sequences of 33 class I tRNAs. *J. Biol. Chem.* **259**: 9461-9471.

Gupta R (1986) Transfer RNAs of Halobacterium volcanii: Sequences of five leucine and three serine tRNAs. *Syst. Appl. Microbiol.* **7**: 102-105.

Gutierrez MC, Kamekura M, Holmes ML, Dyall-Smith ML, Ventosa A (2002) Taxonomic characterization of *Haloferax* sp. ("*H. alicantei*") strain Aa 2.2: description of *Haloferax lucentensis* sp. nov. *Extremophiles* **6**: 479-483.

Holmes ML, and Dyall-Smith ML (2000). Sequence and expression of a halobacterial β-galactosidase gene. *Molecular Microbiology.* **36**: 114-122.

Holmes ML, Nuttall SD, and Dyall-Smith ML (1991) Construction and use of halobacterial shuttle vectors and further studies on Haloferax DNA gyrase. *J. Bacteriol.* **173**: 3807–3813.

Holmes ML, Scopes RK, Moritz RL, Simpson RJ, Englert C, Pfeifer F, and Dyall-Smith ML (1997) Purification and analysis of an extremely halophilic β-galactosidase from *Haloferax alicantei. Biochim Biophys Acta* **1337**: 276-286.

Inoue H, Nojima H, and Okayama H (1990). High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96**: 23-28.

Kamekura M, Oesterhelt D, Wallace R, Anderson P, and Kushner DJ (1988) Lysis of Halobacteria in Bacto-Peptone by Bile Acids. *Appl Environ Microbiol.* **54**: 990-995.

Kita T, Brown MS, and Goldstein JL (1980) Feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in livers of mice treated with mevinolin, a competitive inhibitor of the reductase. *J. Clin. Invest.* **66**: 1094-1100.

Köhrer C, Sullivan EL, RajBhandary UL (2004) Complete set of orthogonal 21st aminoacyl-tRNA synthetase-amber, ochre and opal suppressor tRNA pairs: concomitant suppression of three different termination codons in an mRNA in mammalian cells. *Nucleic Acid Res.* **32**: 6200-6211.

Kozak M (1989) The scanning model for translation: an update. *J Cell Biol.* **108**:229-241.

Kyrpides NC, Woese CR (1998) Archaeal translation initiation revisited: the initiation factor 2 and eukaryotic initiation factor 2B alpha-beta-delta subunit families. *PNAS USA.* **95**: 3726-3730.

Londei P (2005) Evolution of translational initiation: new insights from the archaea. *FEMS Microbiology Review.* **29**: 185-200.

Nieuwlandt DT, and Daniels CJ (1990) An expression vector for the archaebacterium *Haloferax volcanii. J. Bacteriol.* **172**: 7104–7110.

Palmer JR, and Daniels CJ (1994) A transcriptional reporter for in vivo promoter analysis in the archaeon *Haloferax volcanii. Appl. Environ. Microbiol.* **60**: 3867-3869.

Polacek N & Mankin AS (2005) The ribosomal peptidyl transferase center: structure, function, evolution, inhibition. *Crit Rev Biochem Mol Biol.* **40**: 285-311.

Ramesh V, & RajBhandary UL (2001) Importance of the Anticodon Sequence in the Aminoacylation of tRNAs by Methionyl-tRNA Synthetase and by Valyl-tRNA synthetase in an archaebacterium. *J Biol Chem.* **276**: 3660-3665.

Sambrook J, Fritsch EF, and Maniatis T (1989) Molecular Cloning – A Laboratory Manual. 2nd edition.

Sanger F, Nicklen S, and Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. *PNAS USA.* **74**: 5463-5467.

Sartorius-Neef S, Pfeifer F (2004) In vivo studies on putative Shine-Dalgarno sequences of the halophilic archaeon *Halobacterium salinarum. Mol. Microbiol.* **51**: 579-588.

Shine J, Dalgarno L (1975) Determinant of cistron specificity in bacterial ribosomes. *Nature.* **254**:34-38.

Srinivasan G, James CM, and Krzycki JA. (2002). Pyrrolysine encoded by UAG in Archaea: charging of a UAG-decoding specialized tRNA. *Science.* **296**:1459-1462.

Srinivasan G, Krebs MP, and RajBhandary UL (2006) Translation initiation with GUC codon in the archaeon Halobacterium salinarum: implications for translation of leaderless mRNA and strict correlation between translation initiation and presence of mRNA. *Mol Microbiol.* **59**:1013-24.

Stortchevoi A, Varshney U, RajBhandary UL (2003) Common location of determinants in initiator transfer RNAs for initiator-elongator discrimination in bacteria and in eukaryotes. *J Biol Chem.* **278**: 17672-17679.

Tolstrup N, Sensen CW, Garrett RA, Clausen IG (2000) Two different and highly organized mechanisms of translation initiation in the archaeon *Sulfolobus solfataricus*. *Extremophiles*. **4**: 175-179.

Torarinsson E, Klenk HP, Garrett RA (2005) Divergent transcriptional and translational signals in Archaea. *Environmental Microbiol.* 7:1 47-54.

Varshney U, Lee CP, and RajBhandary UL (1991 ) Direct analysis of aminoacylation levels of tRNAs *in vivo*. Application to studying recognition of *Escherichia coli* initiator tRNA mutants by glutaminyl-tRNA synthetase. *J. Biol. Chem.* **266**: 24712–24718.

Warming S, Costantino N, Court DL, Jenkins NA, Copeland NG (2005) Simple and highly efficient BAC recombineering using *galK* selection. *NAR.* **33**: e36.

White BN and Bayley ST (1972) Methionine transfer RNAs from the extreme halophile, *Halobacterium cutirubrum*. *Biochim Biophys Acta.* **272**: 583-587.

Woese CR, Fox E (1977). Phylogenetic Structure of the Prokaryotic Domain: The Primary Kingdoms. *PNAS USA.* **74**: 5088–5090.

Woods WG and Dyall-Smith ML (1997) Construction and analysis of a recombination-deficient (radA) mutant of Haloferax volcanii. *Mol. Bicrobiol.* **23**: 791-797.

Yatime L, Schmitt E, Blanquet S, Mechulam Y (2004) Functional molecular mapping of archaeal translation initiation factor 2. *J Biol Chem.* **279**: 15984-15993.

**Figures:**

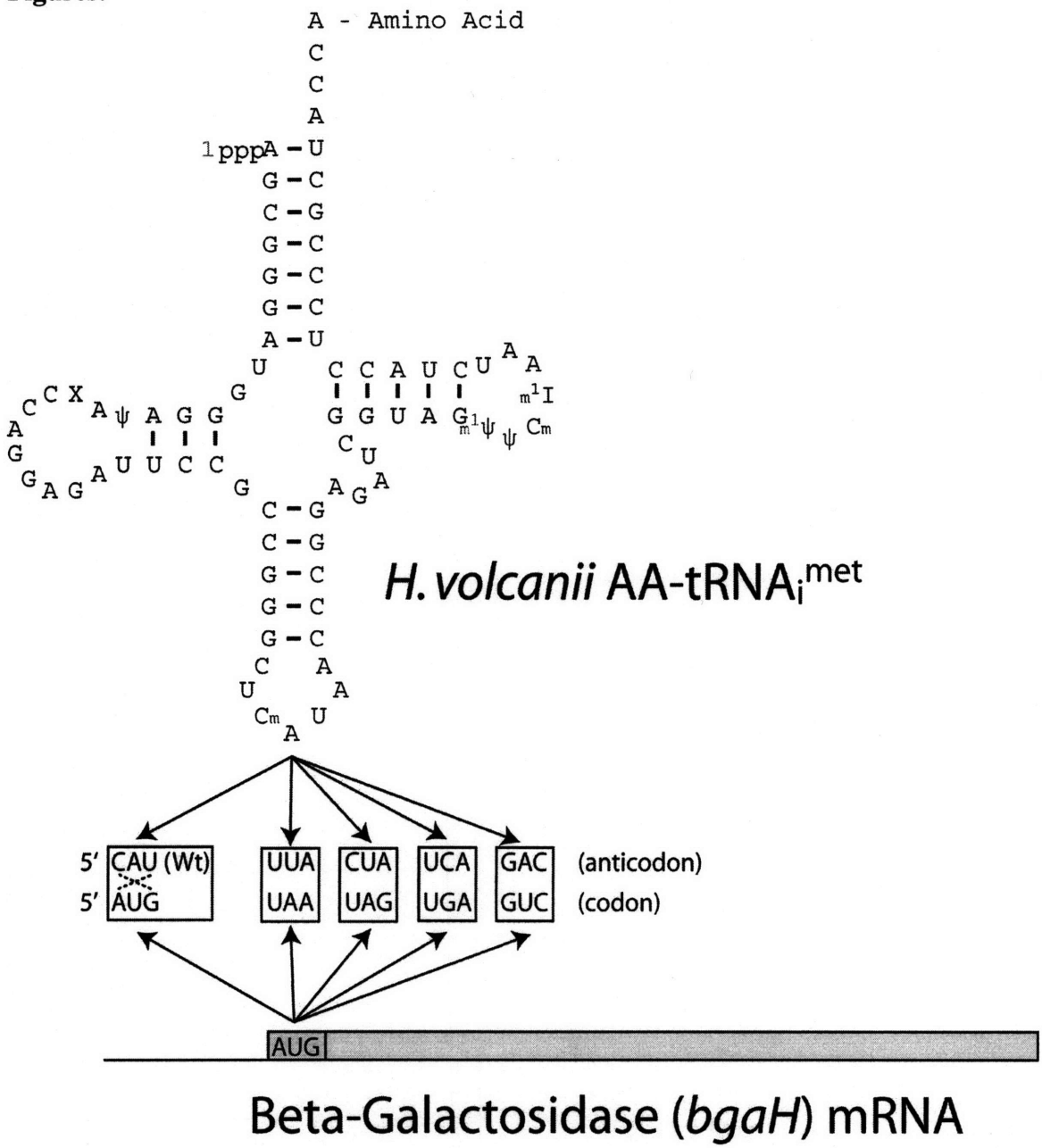


Figure 1. Assay for Translation Initiation. *BgaH* is expressed on pMLH32 in *H. volcanii*. Its start codon is replaced with UAG, UAA, UGA, or GUC. As the wild type initiator tRNA can only decode AUG, there will be no expression, and hence no detectable β-galactosidase activity. However, using pWL201, mutant initiator tRNAs are introduced that can decode each of the respective start codons. When combined, if the tRNA is charged it will produce detectable activity in proportion to how well it functions in translation initiation.

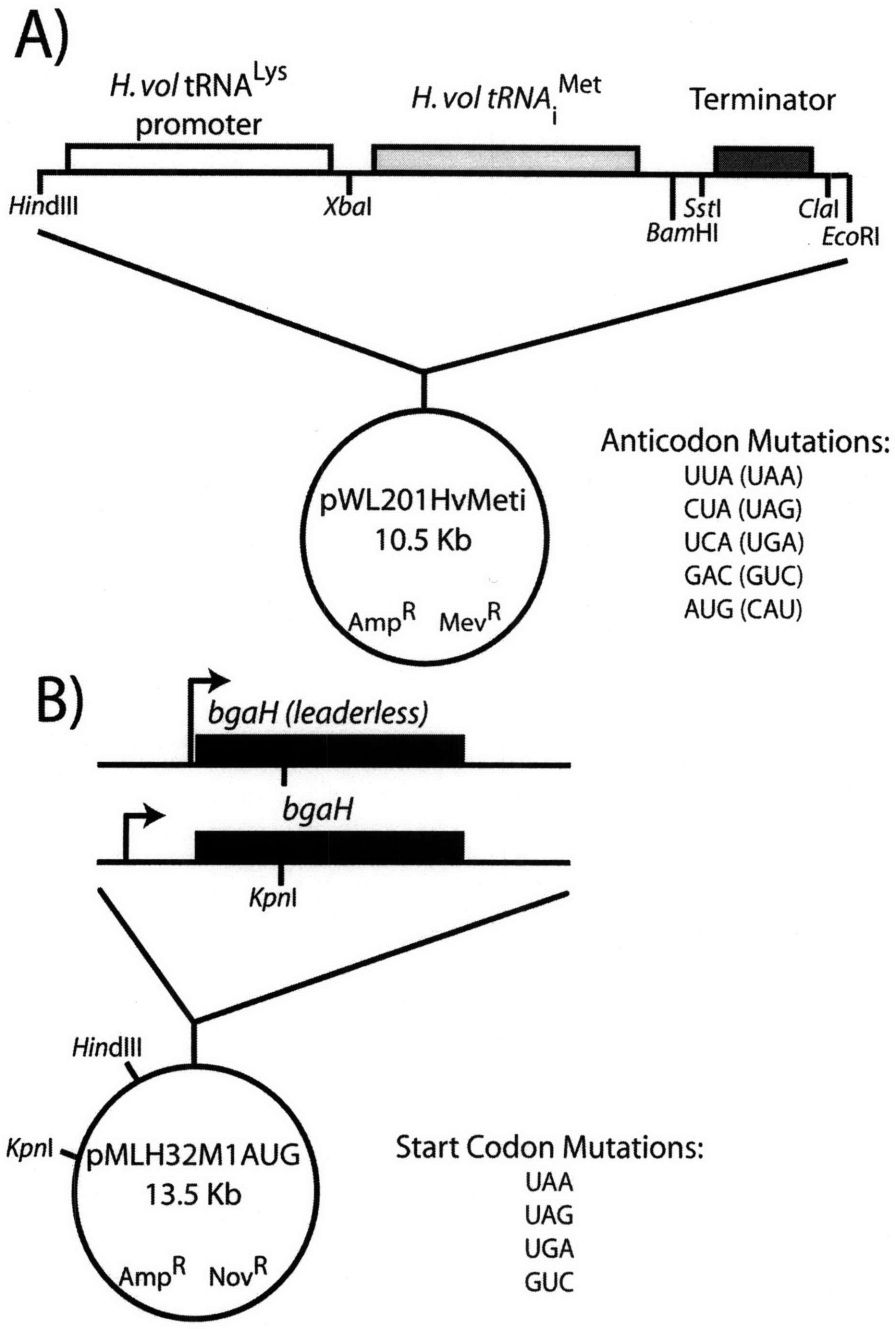


Figure 2. Expression Vectors. (A) The tRNA expression cassette for the *H. volcanii* $tRNA_i^{met}$ derived and $tRNA^{ser}$ derived mutants is ligated into the shuttle vector pWL201. (B) The *bgaH* containing vector pMLH32 is modified such that the reading frame start codon is UAG, UAA, UGA, or GUC. It was created as both leadered and leaderless versions. $Amp^R$, Ampicillin resistance; $Mev^R$, mevinolin resistance; $Nov^R$, novobiocin resistance.

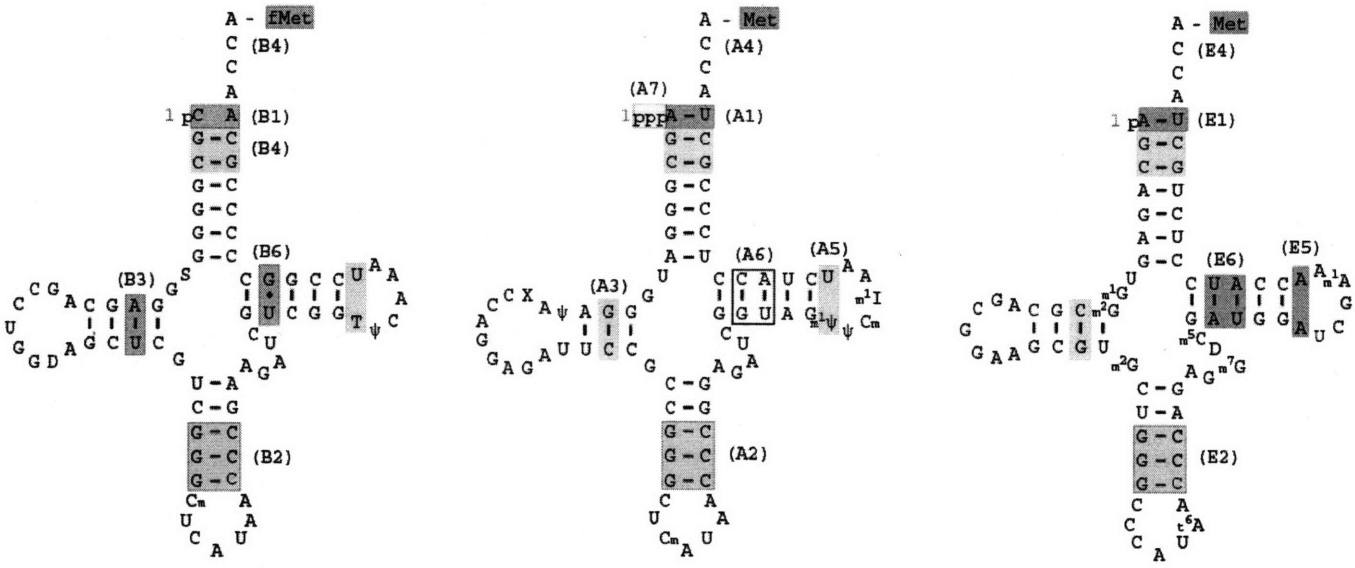


Figure 3. Initiator tRNAs from all three domains of life (bacteria, archaea, & eukarya).
Important in Eubacteria-

B1. Absence of a Watson-Crick base pair at position 1-72
B2. Three consecutive GC base pairs at the bottom of the anticodon stem loop
B3. Presence of a purine11-pyrimidine24 base pair
B4. Methionine Formylation requires 1-72 weak/no base pair, G2C71 & C3G70
B6. U50G64 wobble (modulating function as an elongator)

Important in Eukaryotes-

E1. AU base pair at 1-72
E2. Three consecutive GC base pairs at the bottom of the anticodon stem loop
E4. Not formylated
E5. A54 and A60 in the TψC loop (instead of T54 and pyrimidine60)
E6. Plants/Fungi have a bulky 64 modification; vertebrates have sequence in TψC stem that prevent functioning as an elongator

Present in Archaea-

A1. AU base pair at 1-72
A2. Three consecutive GC base pairs at the bottom of the anticodon stem loop
A3. Presence of a purine11-pyrimidine24 base pair
A4. Not formylated
A5. T54 and pyrimidine60 in the TψC loop
A6. Unknown if the TψC stem is important
A7. 5'ppp in *H. volcanii*

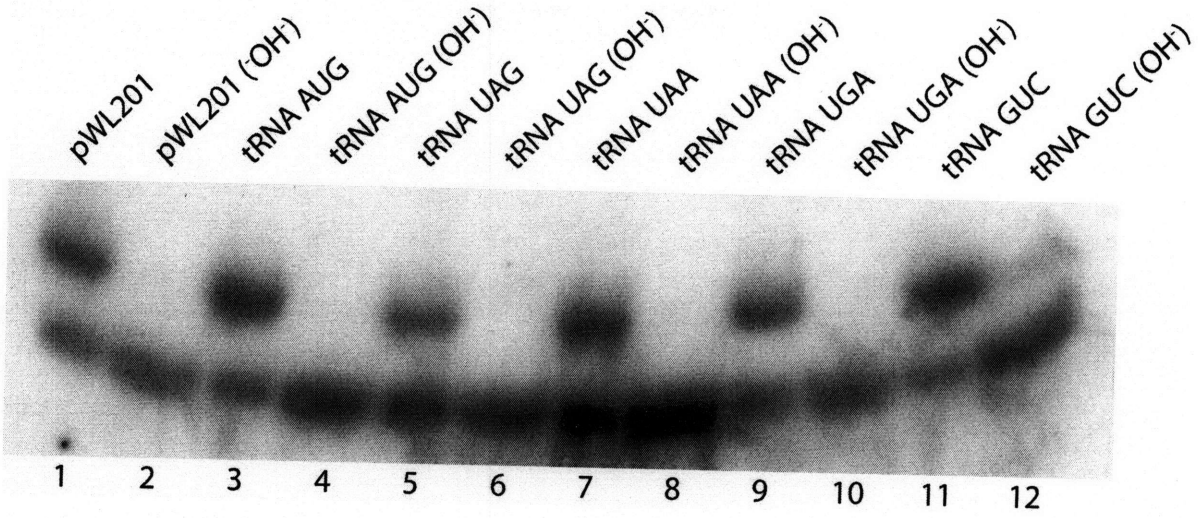


Figure 4. Nonspecific Northern Blotting. Total tRNA was isolated under acidic condition from *H. volcanii* expressing pWLHvMetiAUG, UAG, UAA, UGA, and GUC (*lanes 3-4, 5-6, 7-8, 9-10, 11-12*) or pWL201 (*lanes 1-2*) and subject to northern blotting. Even numbered lanes were subjected to base treatment before loading. Three radio-labeled oligonucleotides which did not target the anticodon were used as probes.

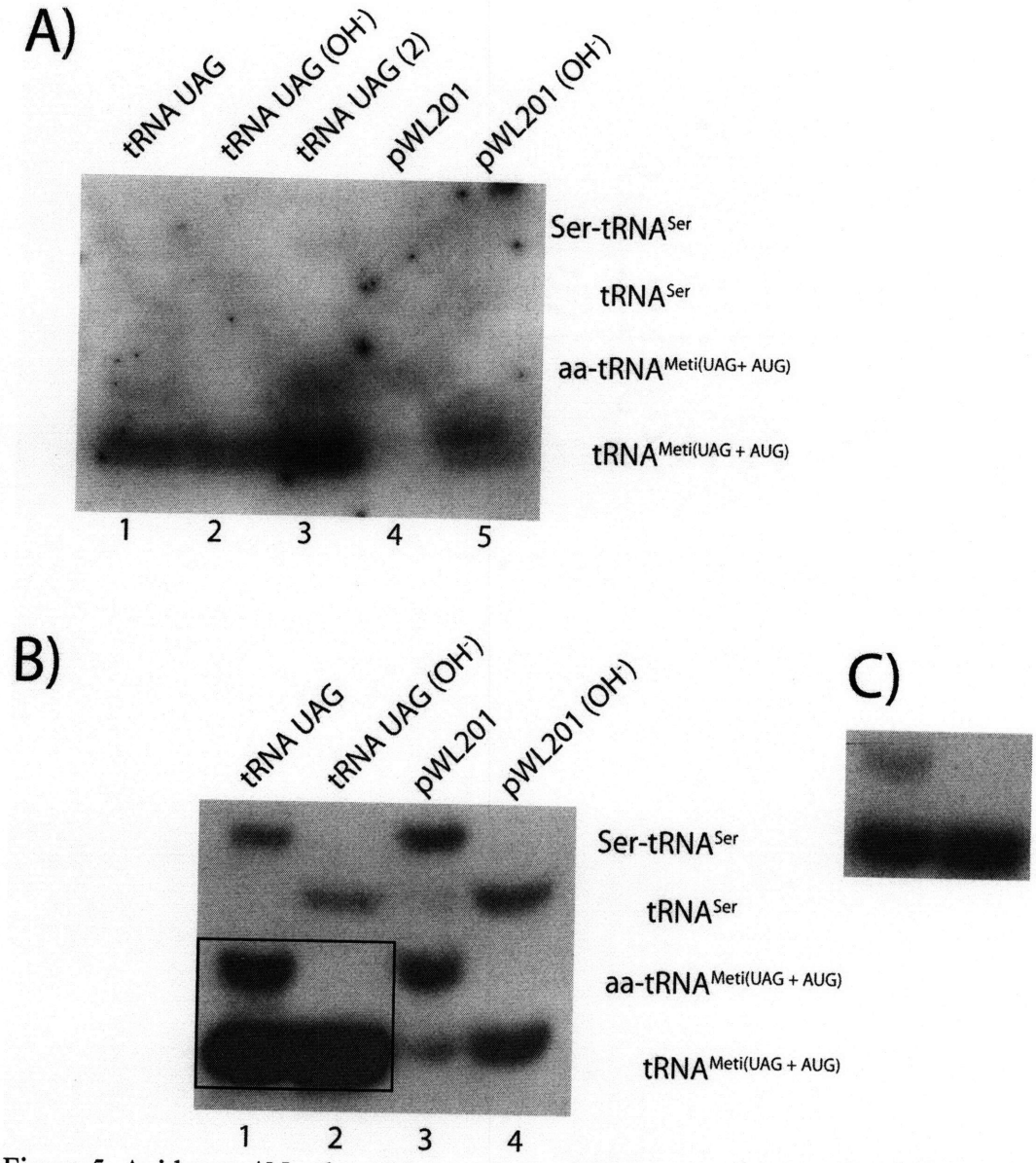


Figure 5. Acid urea / Northern blot analysis of tRNA isolated from *H. volcanii* transformed with pWLHvMet$_i$UAG. (A) Total tRNA was isolated under acidic condition from *H. volcanii* expressing pWLHvMet$_i$UAG (*lanes 1-3*) or pWL201 (*lanes 4-5*) and subject to northern blotting. *Lanes 2* and *5* were subjected to base treatment before loading. (B) Total tRNA was isolated under acidic condition from *H. volcanii*/pWLHvMet$_i$UAG (*lanes 1-2*) or pWL201 (*lanes 3-4*) and subject to northern blotting, with *lanes 2* and *4* base treated. The protocol was improved over part A (see Materials and Methods) (C) The square region in B is shown with less exposure. In all cases, the tRNAs were detected with an oligonucleotide complementary to tRNA$_i^{met}$UAG positions 29-47, and as a control, positions 26-c5 of tRNA$^{ser}$.

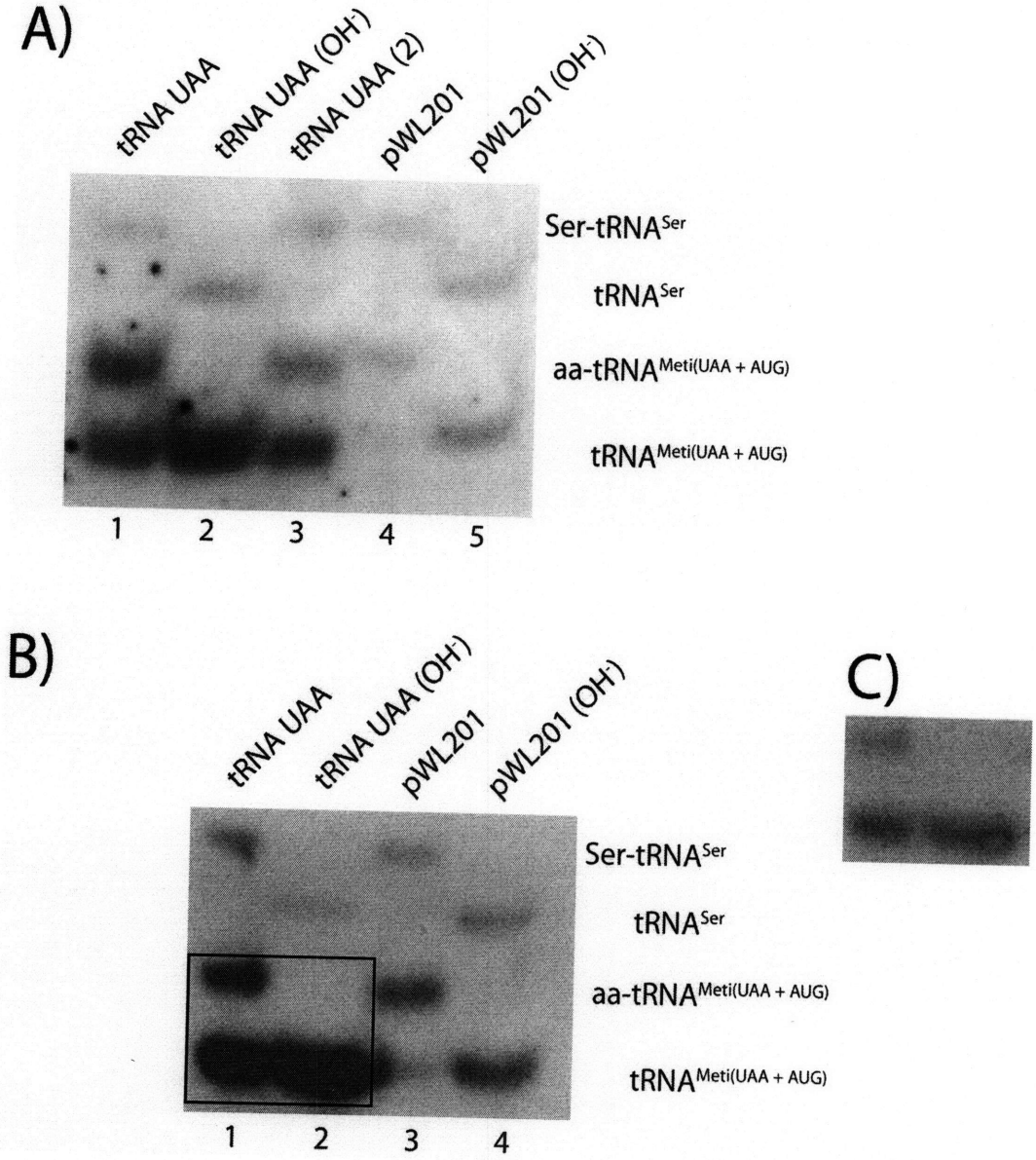


Figure 6. Acid urea / Northern blot analysis of tRNA isolated from *H. volcanii* transformed with pWLHvMet$_i$UAA. For a full description see Figure 5. The only differences are tRNA was isolated from *H. volcanii* expressing pWLHvMet$_i$UAA and the probe was directed against the UAA anticodon.

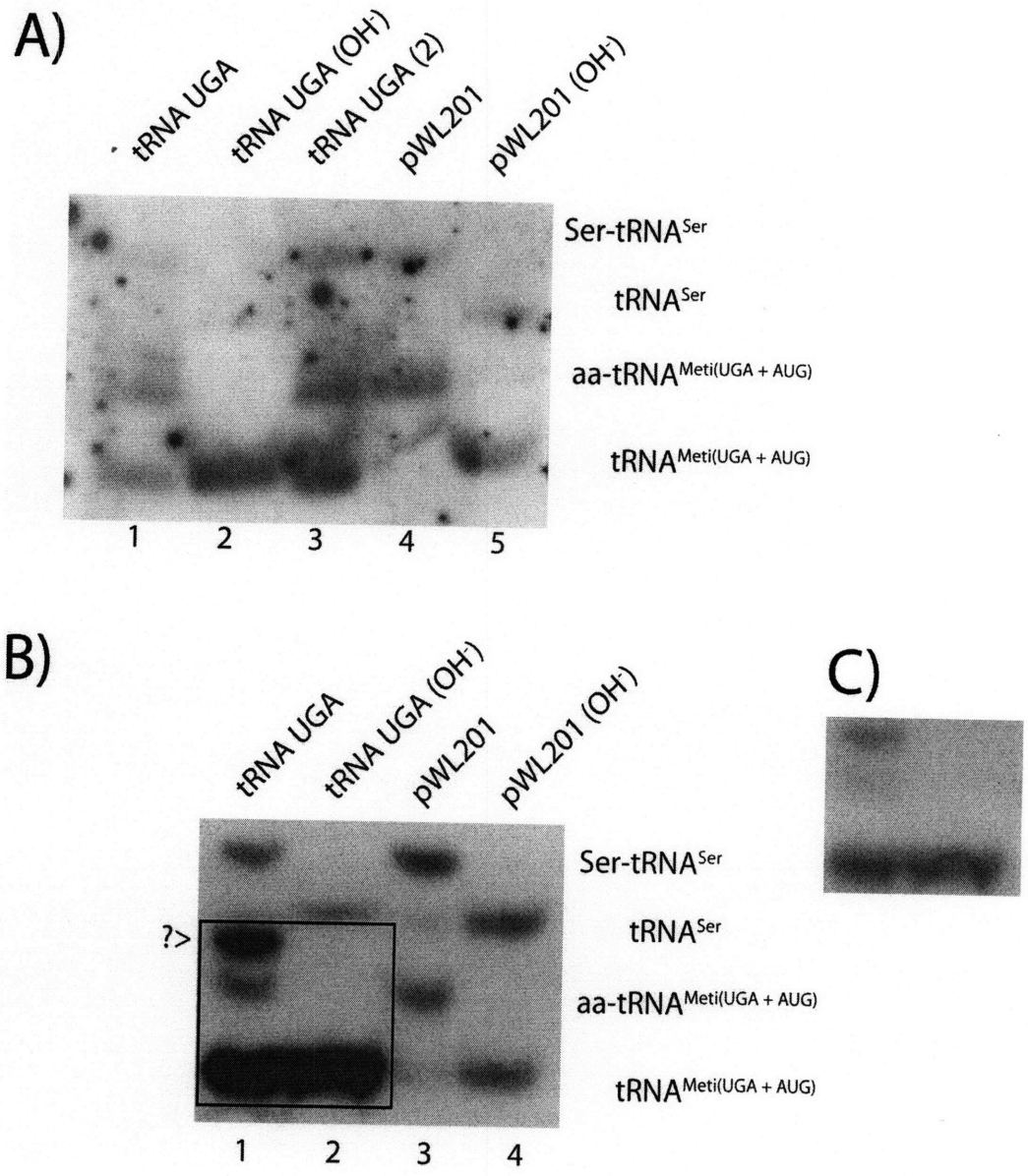


Figure 7. Acid urea / Northern blot analysis of tRNA isolated from *H. volcanii* transformed with pWLHvMet$_i$UGA. For a full description see Figure 5. The only differences are tRNA was isolated from *H. volcanii* expressing pWLHvMet$_i$UGA and the probe was directed against the UGA anticodon.

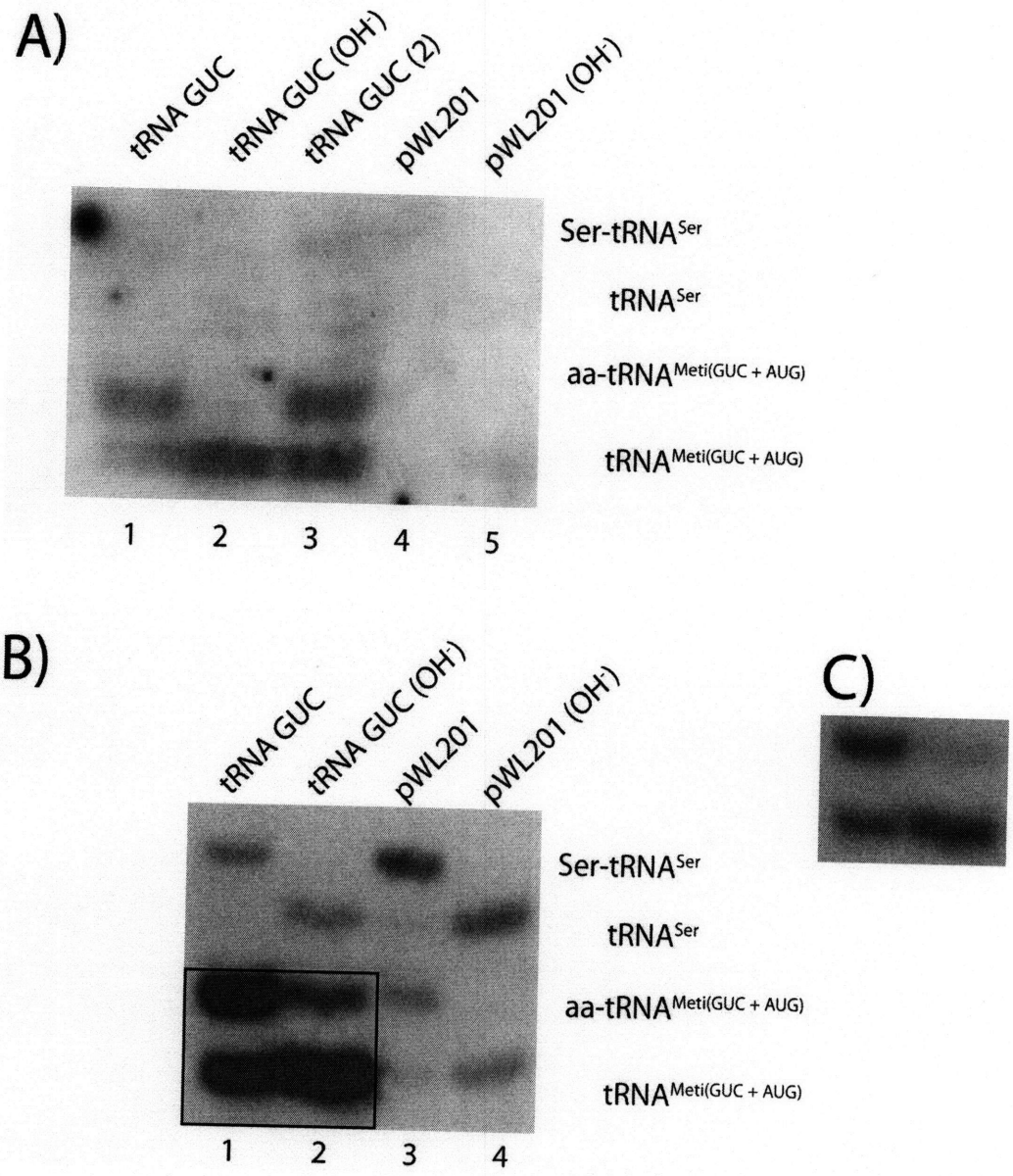


Figure 8. Acid urea / Northern blot analysis of tRNA isolated from *H. volcanii* transformed with pWLHvMet$_i$GUC. For a full description see Figure 5. The only differences are tRNA was isolated from *H. volcanii* expressing pWLHvMet$_i$GUC and the probe was directed against the GUC anticodon.

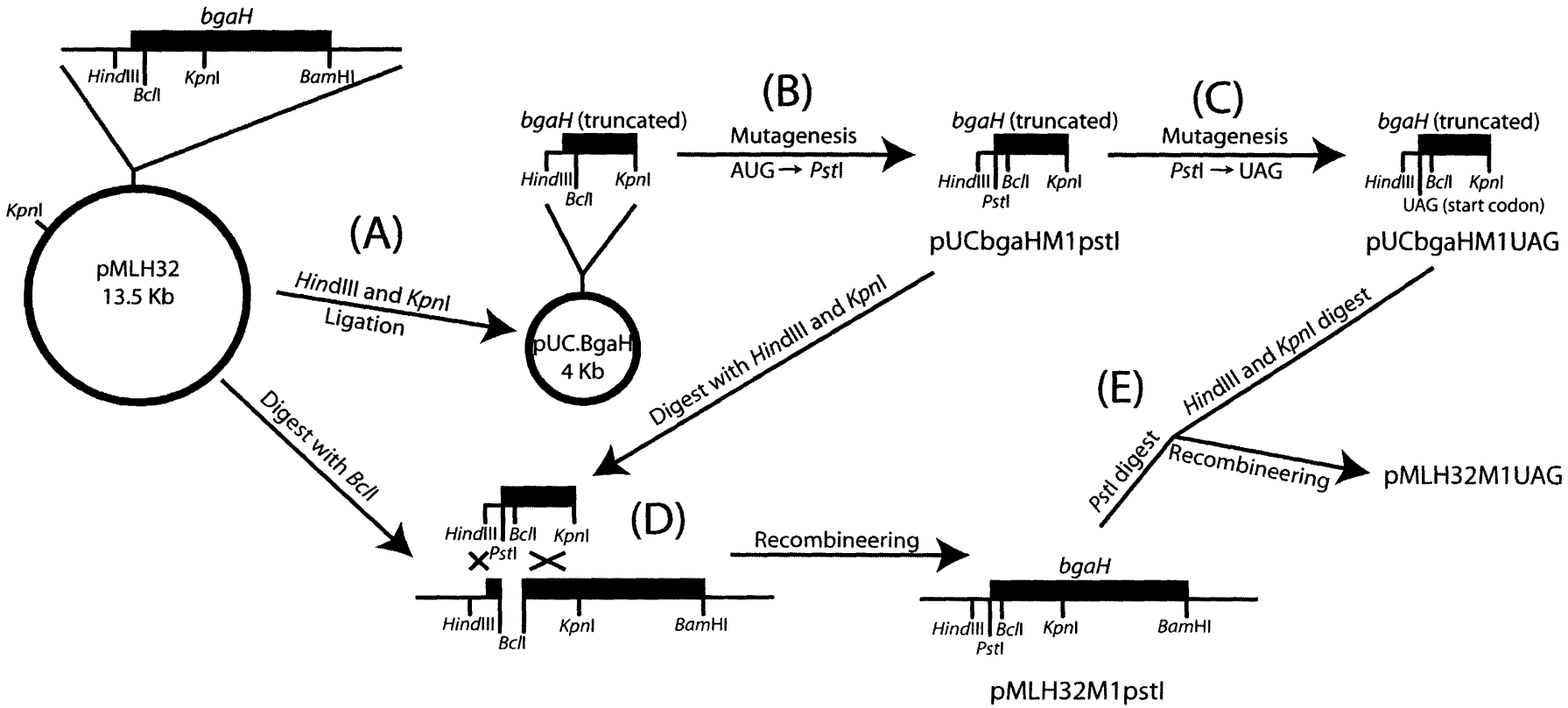


Figure 9. *BgaH* Recombineering Strategy. (A) pMLH32 is digested with *Hin*dIII and *Kpn*I and the *bgaH* fragment is ligated into pUC18. (B) Site directed mutagenesis is done on pUC.bgaH to change the AUG initiation codon to a *Pst*I site. (C) Another round of site directed mutagenesis is done to change the start codon to the desired mutant (also see figure F9 parts A and B). (D) Separately, pMLH32 is digested with *Bcl*I yielding a linear fragment, which is recombineered with the *Hin*dIII/*Kpn*I *bgaH* fragment created in step B. (E) The new pMLH32M1pstI vector is digested with *Pst*I and recombineered with the desired mutant created in step C.

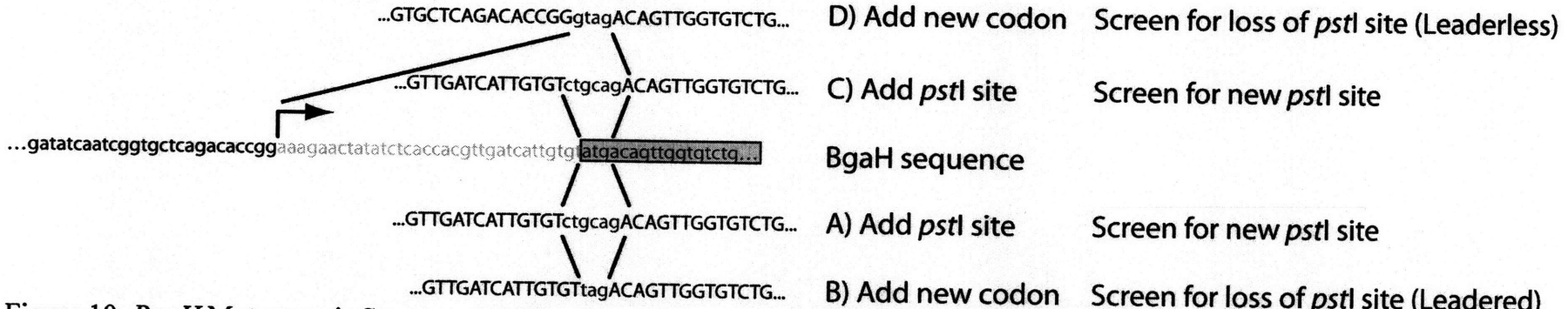


Figure 10. *BgaH* Mutagenesis Strategy. (A) Site directed mutagenesis is used to modify the ATG start codon to CTGCAG (a *Pst*I site). This site is unique in both the pUC.bgaH and pMLH32 constructs, and can easily be screened for. (B) The *Pst*I site is then replaced with the desired start codon by another round of site directed mutagenesis. The resulting plasmids are screened for the loss of the *Pst*I site. (C) Generation of the leaderless reporters use the same strategy as in A, starting with the already created *Pst*I mutant. (D) The second round of site directed mutagenesis removes the 5'UTR region as well as introducing the desired start codon. It is then screened as before.

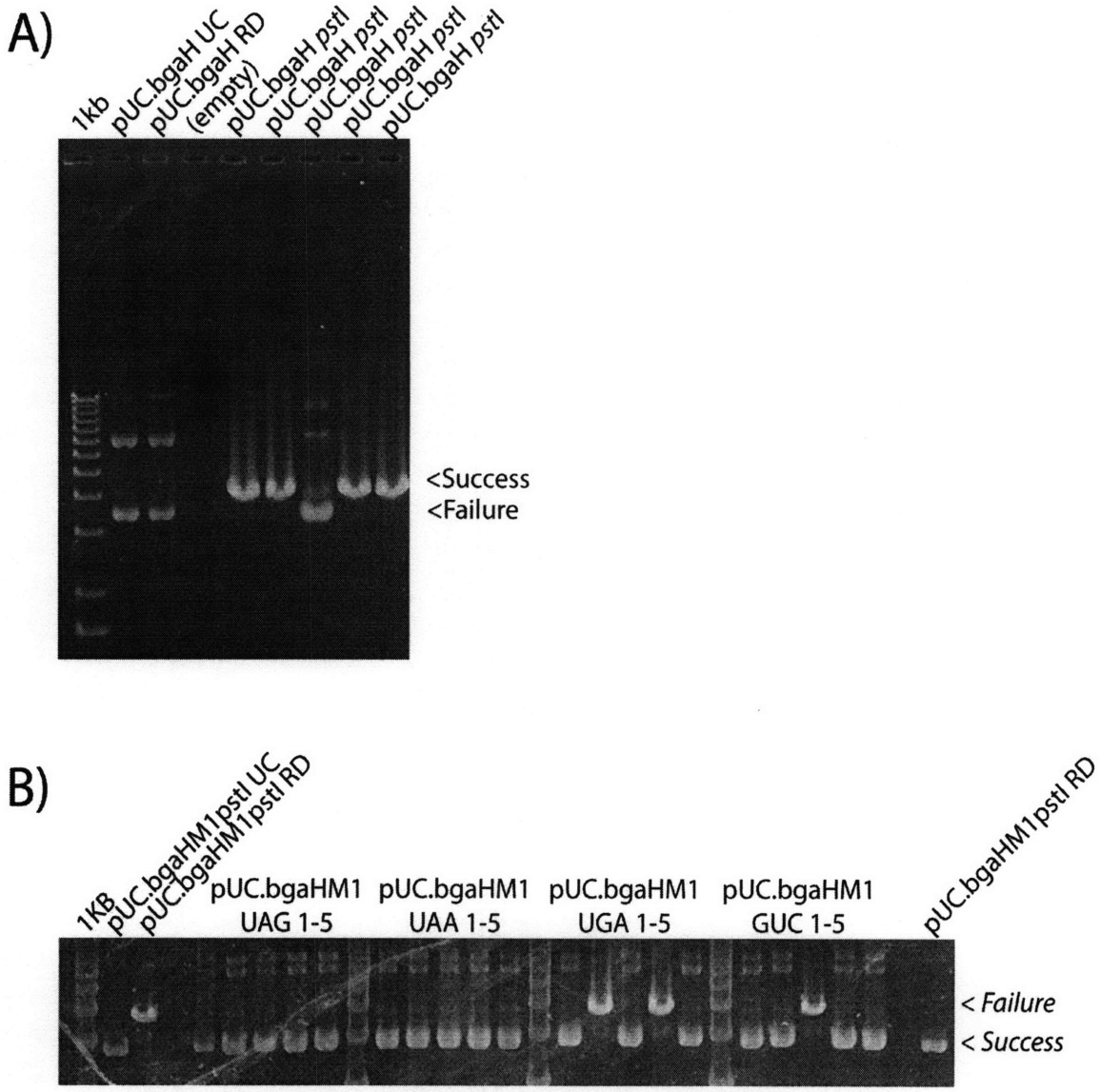


Figure 11. pUCbgaH Mutagenesis Screen (A) Site directed mutagenesis is done on the initiation codon of pUC.bgaH to introduce a *Pst*I site. When digested with *Pst*I, the appearance of a slower migrating (linearized) band indicates successful mutagenesis of that plasmid. The parental vector is run as both uncut (UC) and *Pst*I digested (RD). (B) The second round of mutagenesis introduces UAG, UAA, UGA, or GUC as the start codon. Desired mutants show the opposite response to that in part A when screened with *Pst*I (they remain supercoiled).

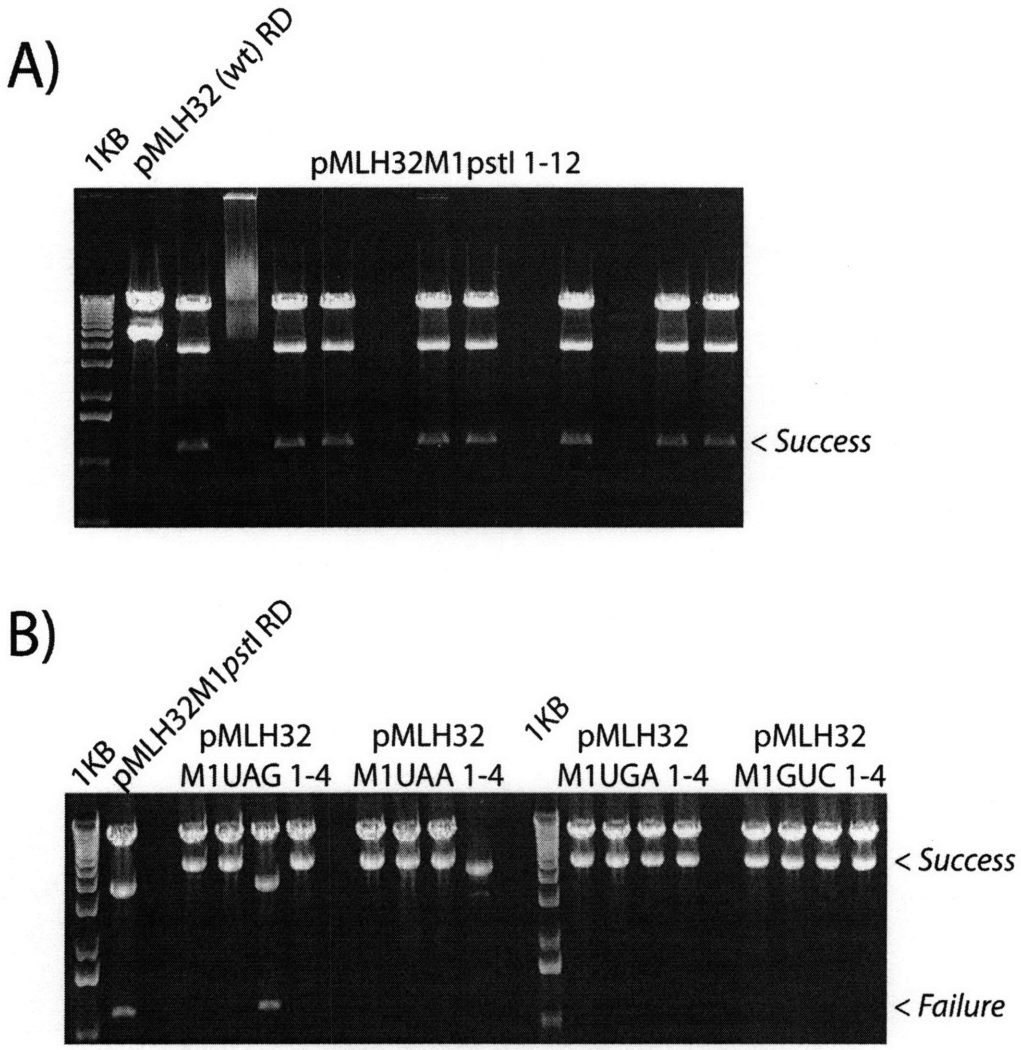


Figure 12. pMLH32 Recombineering Screen. (A) *Bcl*I linearlized pMLH32 is recombined with the *Hin*dIII/*Kpn*I fragment of pUC.bgaHM1pstI. This converts the first codon to a unique *Pst*I site. As the vector is too large to screen with *Pst*I alone, when digested with *Pst*I and *Kpn*I the appearance of a third band indicates successful recombination (*Kpn*I cuts twice, *Pst*I cuts once). The parental vector is also digested (RD), and runs as two bands. (B) The new pMLH32M1pstI vector is linearlized with *Pst*I and recombined with the *Hin*dIII/*Kpn*I fragments of pUC.bgaHM1UAG/UAA/UGA/GUC plasmids. This screen is the opposite of that in A, therefore the loss of the third fragment indicates success when screened with *Pst*I and *Kpn*I.

**Tables:**

Table 1. Comparison of the effects of various buffers on the Beta-Glo assay. The Beta-Glo assay was performed using extracts made from wild-type WFD11 cells (*Background*) or cells expressing *bgaH* from the pMLH32 plasmid (*bgaH*). Reagent was prepared as 1) Standard Beta-Glo buffer, 2) 1:9 Beta-Glo / bgaH Buffer, 3) 1:9 Beta-Glo / 20% sorbitol. (see methods) (A) Cells were assayed directly. (B) Cells were lysed before assay. Note: The media used was Difco Bacto-peptone which is known to cause lysis, so the numbers for the non-lysed cell assay may be artificially high; the readings were beyond the linear range of the equipment; No corrections were made for cellular concentration or background levels.

| A) No Lysis | Buffer 1 | Buffer 2 | Buffer 3 |
|---|---|---|---|
| Background | 11,798 | 36,781 | 33,652 |
| bgaH | 12,329,283 | 6,004,761 | 6,695,211 |

| B) Lysis | Buffer 1 | Buffer 2 | Buffer 3 |
|---|---|---|---|
| Background | 73,008 | 168,984 | 125,109 |
| bgaH | 80,684,613 | 78,225,084 | 32,692,338 |

Table 2. Comparison of the effects of a lysis step on the standard Beta-Glo assay. WFD11 was transformed with pMLH32 based plasmids containing mutant initiation codons (*bgaH*), and with that plasmid and pWL201 based plasmids expressing the complementary tRNA (*bgaH + tRNA*). The AUG row contains pMLH32 without and with overexpressed wildtype initiator tRNA. The Beta-Glo assay with the standard buffer was performed on the cells. (A) Cells were assayed directly. (B) Cells were lysed before assay. Note: the readings for AUG were beyond the linear range of the equipment; No corrections were made for cellular concentration. Cells were assayed at stationary phase.

| A) No Lysis | bgaH | bgaH + tRNA | Fold Increase | Activity (%) |
|---|---|---|---|---|
| Background | 23,513 | | - | 0% |
| UAG | 29,001 | 24,763 | -15% | 0% |
| UAA | 27,103 | 32,033 | 18% | 0% |
| UGA | 23,106 | 28,741 | 24% | 0% |
| GUC | 33,114 | 276,167 | 734% | 3% |
| AUG | 7,513,082 | 8,911,819 | 19% | 100% |

| B) Lysis | bgaH | bgaH + tRNA | Fold Increase | Activity (%) |
|---|---|---|---|---|
| Background | 348,444 | | - | 1.59% |
| UAG | 348,444 | 337,757 | -3% | 1.54% |
| UAA | 335,498 | 371,745 | 11% | 1.70% |
| UGA | 354,164 | 357,872 | 1% | 1.64% |
| GUC | 357,017 | 804,686 | 125% | 3.68% |
| AUG | 17,247,087 | 21,872,403 | 27% | 100.00% |

Table 3. Using $OD_{600}$ correction on the standard Beta-Glo assay with a lysis step. WFD11 was transformed with pMLH32 based plasmids containing mutant initiation codons (*bgaH*), and with that plasmid and pWL201 based plasmids expressing the complementary tRNA (*bgaH + tRNA*). The AUG row contains pMLH32 without and with overexpressed wildtype initiator tRNA. The Beta-Glo assay with the standard buffer was performed on lysed cells, measured in duplicates, and performed twice on the same samples. (A) No correction for cellular concentration was made. (B) Values were divided by the $OD_{600}$ of the cells. Note: all readings were within the linear range of the equipment; Cells were assayed at mid log phase (0.3-0.8 $OD_{600}$).

A) No $OD_{600}$ correction

| | bgaH | bgaH + tRNA | Fold Increase | Activity (%) |
|---|---|---|---|---|
| UAG | 104,006 | 121,376 | 17% | 1% |
| UAA | 114,035 | 125,987 | 10% | 1% |
| UGA | 113,580 | 118,778 | 5% | 1% |
| GUC | 135,623 | 4,021,364 | 2865% | 20% |
| AUG | 44,773,918 | 20,380,025 | -54% | 100% |

B) With $OD_{600}$ correction

| | bgaH | bgaH + tRNA | Fold Increase | Activity (%) |
|---|---|---|---|---|
| UAG | 319,038 | 90,783 | -72% | 0% |
| UAA | 178,179 | 163,195 | -8% | 0% |
| UGA | 236,133 | 153,459 | -35% | 0% |
| GUC | 376,731 | 4,958,526 | 1216% | 8% |
| AUG | 124,718,434 | 63,292,004 | -49% | 100% |

## Table 4. Strains and Plasmids

| Strain: | Description: | Source: |
|---|---|---|
| XL1-Blue | *F'::Tn10 proA+B+ lacIq Δ(lacZ)M15/ recA1 endA1 gyrA96 (NaIr) thi hsdR17 (rk-mk+) glnV44 relA1 lac* | |
| GM2163 (*dam*⁻) | F⁻ *ara*-14 *leuB6 thi*-1 *fhuA31 lacY1 tsx*-78 *galK2 galT22 supE44 hisG4 rpsL136 (Strr) xyl-5 mtl-1 dam13::Tn9 (Camr) dcm-6 mcrB1 hsdR2 (rK–mK+) mcrA* | NEB |
| ER2925 (*dam*⁻) | As above, with *endA1* | NEB |
| DH10B | *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Δ*lacX74 deoR endA*1 *araD*139 Δ(*ara, leu*)7697 *rpsL recA1 nupG* Φ80d*lacZ*ΔM15 *galU galK* | |
| SW102 | DH10B [λ*c*1857 (*cro-bioA*)<>*Tet*] ΔgalK | Warming *et al.*, 2005 |
| *H. Volcanii WFD11* | | Doolittle WF |
| *H. Volcanii DS52* | WFD11 ΔradA | Dyall-Smith M |

| Plasmid: | Description: | Source: |
|---|---|---|
| **pUC tRNAs** | | |
| pUCsptProM | an archaeal expression cassette with a yeast $tRNA^{Pro}$ | - |
| pUCsptHvMetI | as above, but with the *H. volcanii* initiator tRNA | Ramesh & RajBhandary, 2001 |
| pUCsptHvMetIUAG | as above, but potentially able to decode UAG (CUA anticodon) | Ramesh & RajBhandary, 2001 |
| pUCsptHvMetIUAA | as above, but potentially able to decode UAA (UUA anticodon) | this work |
| pUCsptHvMetIUGA | as above, but potentially able to decode UGA (UCA anticodon) | this work |
| pUCsptHvMetIGUC | as above, but potentially able to decode GUC (GAC anticodon) | Ramesh & RajBhandary, 2001 |
| pUCsptHvSerUAG | as pUCsptHvMeti, but expressing the *H. volcanii* $tRNA_3^{ser}$ modified to decode UAG (CUA anticodon) | This work[2] |
| pUCsptHvSerUAA | as above, but modified to decode UAA (UUA anticodon) | This work[2] |
| pUCsptHvMetIGUC-ACSL | as pUCsptHvMetiGUC, but with the C30,A31,U39,G40 | This work[2] |
| pUCsptHvMetIGUC-T72C | as pUCsptHvMetiGUC, but with U72 | This work[2] |
| **pWL tRNAs** | | |
| pWL201 | *Escherichia coli / Haloferax volcanii* shuttle vector | Lam and Doolittle, 1989 |
| pWL.HvMeti | pWL201 with the sptHvMeti expression cassette | Ramesh & RajBhandary, 2001 |
| pWL.HvMetiUAG | as above, but able to decode UAG | Ramesh & RajBhandary, 2001 |
| pWL.HvMetiUAA | as above, but able to decode UAA | this work[1] |
| pWL.HvMetiUGA | as above, but able to decode UGA | this work[1] |
| pWL.HvMetiGUC | as above, but able to decode GUC | Ramesh & RajBhandary, 2001 |
| pWL.HvSerUAG | pWL201 with the sptHvSer expression cassette - modified to decode UAG | This work[2] |
| pWL.HvSerUAA | as above, but modified to decode UAA | This work[2] |
| **pUC bgaH** | | |
| pUC.bgaH | pUC18 carrying the *Hind*III/*Kpn*I fragment of pMLH32 | Srinivasan G |
| pUC.bgaHM1pstI | as above, but with the first *bgaH* codon repaced w/ a pstI site (CTGCAG) | This work |
| pUC.bgaHM1UAG | as pUC.bgaH, but with UAG as the first codon | This work |
| pUC.bgaHM1UAA | as pUC.bgaH, but with UAA as the first codon | This work |
| pUC.bgaHM1UGA | as pUC.bgaH, but with UGA as the first codon | This work |
| pUC.bgaHM1GUC | as pUC.bgaH, but with GUC as the first codon | This work |

| | | |
|---|---|---|
| pUC.bgaHM1AUGL | as pUC.bgaH but lacking the 5'UTR | This work |
| pUC.bgaHM1UAGL | as pUC.bgaHM1UAG but lacking the 5'UTR | This work |
| pUC.bgaHM1UAAL | as pUC.bgaHM1UAA but lacking the 5'UTR | This work |
| pUC.bgaHM1UGAL | as pUC.bgaHM1UGA but lacking the 5'UTR | This work |
| pUC.bgaHM1GUCL | as pUC.bgaHM1GUC but lacking the 5'UTR | This work |
| | | |
| pUC.bgaHS184pstI | as pUC.bgaH, but with the 184 codon (serine) repaced w/ a pstI site | This work |
| pUC.bgaHS184UAG | as above, but w/ UAG at position 184 | This work |
| pUC.bgaHS184UAA | as above, but w/ UAA at position 184 | This work |
| | | |
| **pMLH32 bgaH** | | |
| pMLH32 | *Escherichia coli / Haloferax volcanii* shuttle vector containing *bgaH* | Holmes, 2000 |
| pMLH32M1pstI | as above, but with the first *bgaH* codon repaced w/ a pstI site (CTGCAG) | This work |
| pMLH32M1UAG | as pMLH32, but with UAG as the first codon | This work |
| pMLH32M1UAA | as pMLH32, but with UAA as the first codon | This work |
| pMLH32M1UGA | as pMLH32, but with UGA as the first codon | This work |
| pMLH32M1GUC | as pMLH32, but with GUC as the first codon | This work |
| | | |
| pMLH32M1AUGL | as pMLH32 but lacking the 5'UTR | This work |
| pMLH32M1UAGL | as pMLH32M1UAG but lacking the 5'UTR | This work |
| pMLH32M1UAAL | as pMLH32M1UAA but lacking the 5'UTR | This work |
| pMLH32M1UGAL | as pMLH32M1UGA but lacking the 5'UTR | This work |
| pMLH32M1GUCL | as pMLH32M1GUC but lacking the 5'UTR | This work |
| | | |
| pMLH32S184pstI | as pMLH32, but with the 184 codon (serine) repaced w/ a pstI site | This work |
| pMLH32S184UAG | as above, but w/ UAG at position 184 | This work |
| pMLH32S184UAA | as above, but w/ UAA at position 184 | This work |

[1]The second *Sst*I site was mutated to a *Cla*I site (see methods)

[2]Seven nucleotides were lost immediately 3' of the tRNA (see methods)

Table 5. Media and Reagents

**Standard *E.coli*:**

LB, per liter:

10g tryptone, 5g yeast extract, 10g NaCl
for solid media, 15g agar per liter

**The Halohandbook – *H. Volcanii* transformation:**

30% Salt Water Stock, per liter:

30g $MgCl_2$-$6H_2O$, 17g MgSO4, 7g KCl, 5ml of 1M $CaCl_2$, pH 7.5 w/ Tris-Cl Buffer

18% MGM, per liter:

600ml 30% Salt Water Stock, 5g Oxoid Peptone, 1g Yeast Extract, pH 7.5 w/ Tris Base
for solid media, 15g agar per liter

Buffered Spheroplasting Solution:

1.0M NaCl, 27mM KCl, 50mM Tris-HCl (pH 8.2), 15% sucrose, 15% glycerol

Unbuffered Spheroplasting Solution:

1.0M NaCl, 27mM KCl, 15% sucrose

60% PEG Buffer:

60% polyethylene glycol-600 w/v in Unbuffered Spheroplasting Solution

**Cline, *et al*, 1989 - *H. Volcanii* transformation:**

*H. volcanii* Growth Medium, per liter:

125g NaCl, 45g $MgCl_2$-$6H_2O$, 4.9g MgSO4, 10g KCl, 1.34g $CaCl_2$-$2H_2O$, 3g yeast extract, 5g tryptone

Spheroplasting Buffer:

0.8M NaCl, 30mM KCl, 50mM Tris-HCl (pH 8.3), 15% sucrose, 15% glycerol

Speroplasting Dilution Buffer:

3.4M NaCl, 30mM KCl, 50mM Tris-HCl (pH 7.2), 175mM $MgSO_4$, 5mM $CaCl_2$, 15% sucrose

60% PEG Buffer:

60% polyethylene glycol-600 w/v in Speroplasting Buffer

*H volcanii* Solid medium, per liter:

50mM Tris-HCl (pH 7.2), 180g NaCl, 43g $MgSO_4$, 2.5g KCl, 0.7g $CaCl_2$-$2H_2O$, 3g yeast extract, 5g tryptone, 15g agar

**Acid Urea/Northern:**

Acid Urea PAGE:

7M Urea, 6.5% 19:1 acrylamide/bis, 0.1M NaOAc (pH 5), [40x20x15cm]

Transfer Buffer:

10mM Tris-OAc (pH 7.8), 5mM NaOAc, 0.5mM EDTA

SSC, for 1X:

150mM NaCl, 14mM NaCitrate (pH 7.0)

Denhardts Solution:

0.02% polyvinylpyrrolidone 40, 0.02% BSA, 0.02% Ficoll

**BgaH ONPG / Beta-Glo assay:**

BgaH buffer:

2.5M NaCl, 50mM Tris-HCl (pH 7.2), 10μM $MnCl_2$, 0.1% BME* (store at 4° w/o BME)