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Can The Insertion Of An E. coli Shine-Dalgarno Sequence Upstream Of M. ruber proA Of The proBA Operon Enhance Its Expression, As Measured By A Complementation Assay Using E. coli Null Strains?

Matthew D. Murphy
Augustana College - Rock Island

Dr. Lori Scott
Augustana College - Rock Island

Can the insertion of an *E. coli* Shine-Dalgarno sequence upstream of *M. ruber* proA of the proBA operon enhance its expression, as measured by a complementation assay using *E. coli* null strains?

Matthew Murphy and Lori Scott

Augustana College

Rock Island, IL 61201

Problem and Purpose:

In previous work, students in Dr. Scott's BIOL375/Molecular Genetics course (winter 2013-14 and winter 2014-15) used gene annotation and functional genomics to elucidate the steps in the proline biosynthesis pathway of the thermophile *Meiothermus ruber* by comparing this system to the model organism *Escherichia coli* K12 MG1655. In Phase 1 of the project, ORFs with locus tags Mrub_1080, Mrub1079 and Mrub_1345 were found to possess many structural and functional features in common with *E. coli* proB, proA and proC, respectively, by comparing the two systems using publically available bioinformatics tools linked to the GENI-ACT platform (*e.g.,* NCBI BLAST, CDD, PFam, TIGRfam, to name a few). In Phase 2, the *M. ruber* genes were tested for their ability to complement the appropriate *E. coli* Keio null strains (Baba et al., 2006) using the system described by the GENI-Science group (GENI-Science.org, 2015). Mrub_1079 and Mrub_1345 complemented their *E. coli* null strain counterparts, but Mrub_1080 did not. Also tested for complementation was an *M. ruber* proBA clone, which failed to complement in either the *E.coli* proB null or *E. coli* proA null strains.

In *E. coli*, the proB and proA genes form an operon (Deutch *et al.,* 1984). The two enzymes aggregate into a multimeric bi-functional enzyme complex known as prolinemulti-cplx; proA is required for the proper functioning of the proB, but not the reverse (Smith *et al.,* 1984). Annotation evidence suggests the same conditions apply to the *M. ruber* system. We propose two reasons why complementation failed for the *M. ruber* proB gene: 1) the interspecies proBA protein complex does not function in the null strain; and 2) the *M. ruber* operon is missing a Shine-Dalgarno site in front of the proA mRNA. A Shine-Dalgarno sequence is found upstream of the *E.coli* proA gene in the proBA operon, but the same sequence is not found upstream of the *M. ruber* proA gene of the proBA clone. In this study, we used site-directed mutagenesis to

create an *E.coli* Shine-Dalgarno sequence upstream of the *M. ruber* proA gene of the proBA clone, which we hypothesized would result in complementation of the *M. ruber* proB gene in *E. coli* Keio proB- null strain.

Background:

In this study, *E. coli* K12 MG1655 was the model system. The steps, genes and enzymes involved in proline biosynthesis of *E. coli* K12 MG1655 are displayed in Figure 1. In E. coli K12 MG1655, γ-glutamyl kinase (GK, proB) catalyzes the L-glutamate to L-glutamyl-5-phosphate reaction; glutamate-5-semialdehyde dehydrogenase (GSD, proA) catalyzes the next step to L-glutamate-5-semialdehyde, which spontaneously converts to 1-pyrroline-5-carboxylate; and pyrroline-5-carboxylate reductase (P5CR, proC) catalyzes the final reaction to L-proline (Smith *et al*., 1984). GK and GSD form an enzyme complex, but P5CR functions independently.

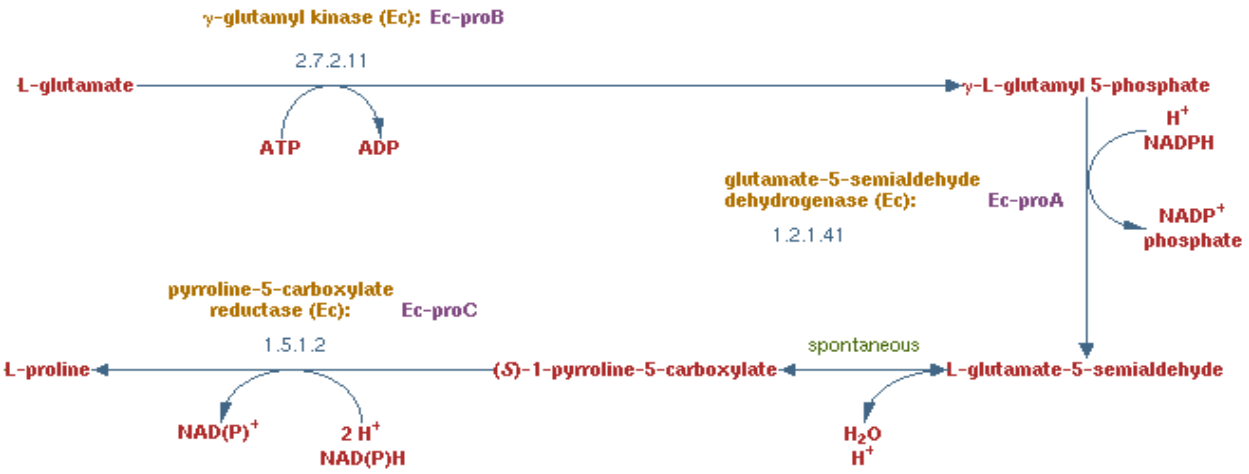

Figure 1. Proline biosynthesis pathway in *E. coli* K12 MG1655. The enzymes in this figure are highlighted gold to indicate that functional evidence is available; E.C numbers are provided below each enzyme name (Metacyc Pathway: L-proline biosynthesis I). The image was taken from the proline biosynthesis page on Metacyc.org (http://ecocyc.org/ECOLI/NEW-IMAGE?type=PATHWAY&object=PROSYN-PWY)

The functional studies supporting the function of GK and GSD are these. A study performed by Baich in 1969, "Proline synthesis in Escherichia coli. A proline-inhibitable glutamic acid kinase", showed evidence of a negative feedback loop by proline inhibition of the glutamic acid kinase, a glutamic acid-dependent reaction that is responsible for the dephosphorylation of ATP for the conversion of glutamate to glutamyl-5-phosphate (Baich 1969). A second study by Baich in 1971, "The biosynthesis of proline in Escherichia Coli: phosphate-dependent glutamate-semialdehyde dehydrogenase (NADP), the second enzyme in the pathway", showed evidence of a phosphate-dependent functionality of the second enzyme in the proline biosynthesis pathway, glutamate-semialdehyde dehydrogenase, in converting glutamyl-5-phosphate to glutamate-5-semialdehyde (Baich 1971).

The proB and proA genes in *E. coli* K12 MG1655 are contained within a operon under the control of a single genetic promoter region and are thus transcribed and translated as a continuous piece of DNA (Deutch et al., 1984). Figure 2 was constructed from two images taken from the JGI IMG/edu platform for proB (locus tag b0242), specifically the Gene Context page and Gene Neighborhood page. The intergenic space between the two genes has a notable feature termed a Shine-Dalgarno sequence. This sequence serves as a ribosome binding site, or an indicator to ribosomes to bind to the resulting mRNA after transcription of the DNA to induce protein synthesis.

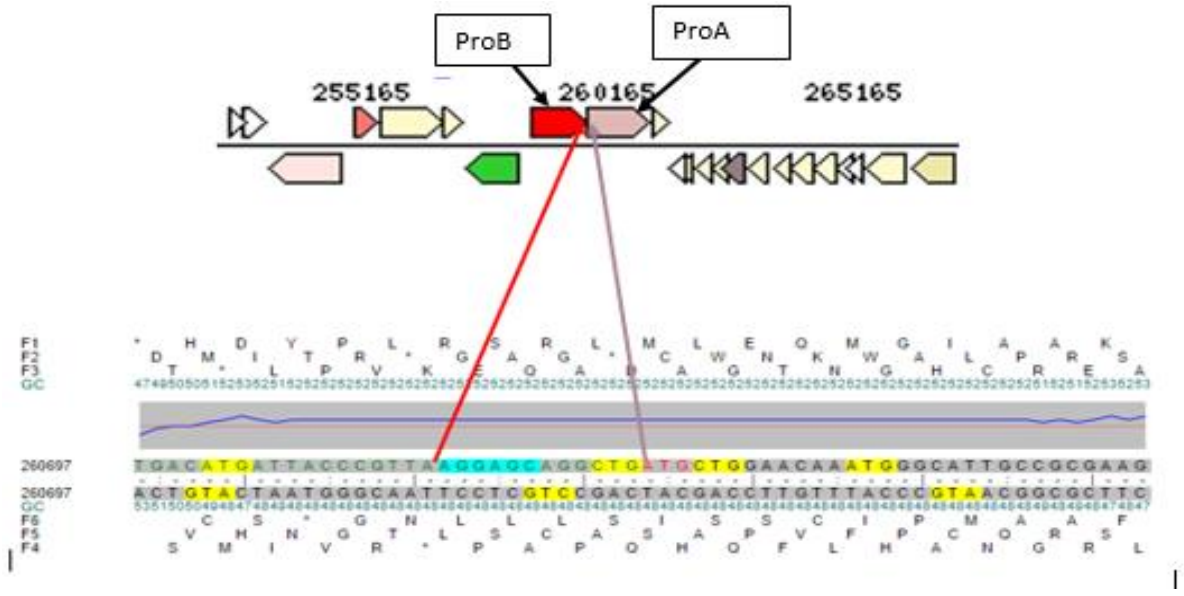

Figure 2. Chromosome region containing the proB and proA genes of *E. coli* K12 MG1655, which shows chromosome coordinates and gene order (top panel) and the intergenic nucleotide sequence (bottom panel) between proB and proA. Top panel: genes are indicated by broad arrows, which point in the direction of transcription. The vertical arrows between top and bottom panels indicate the stop codon of proB and the start codon of proA. Bottom panel: the blue sequence is the Shine-Dalgarno sequence upstream of the proA gene; the red ATG is the proA start codon. Both images were taken from IMG/edu.org and accessed through the Gene Details pages for *E. coli* proB (locus tag b0242) and proA (locus tag b0243).

Bacteria are one of the most prolific and versatile organisms on planet Earth. The varieties are countless, their functions are innumerable, their numbers are unfathomable, and some of their living environments would be thought to be unlivable (Speer and Waggoner, 2006). Unfortunately, much of what we know about bacteria and their cellular processes come from the studies of relatively few species. The goal of the Department of Energy Joint Genome Institute's Genome Encyclopedia of Bacteria and Archaea (GEBA) project is to expand our understanding of bacteriaby expanding our knowledge across the prokaryotic portion of Tree of Life (Wu et al., 2009). We may find new and relevant processes that we can exploit. Toward that end, our project studies the GEBA organism *Meiothermus ruber* DSM1279. *M. ruber* has these features: Gram-negative, nonmotile rods, 3 to 6 by 0.5 to 0.8 μm, obligately thermophilic,

and non-spore-forming. On potato-peptone-yeast extract medium, the temperature range for growth is approx. 35-70°C, with an optimum temperature at 60°C (Loginova *et al*., 1984). Its genome was sequenced by Tindall *et al.* (2010). The circular genome is composed of 3,097,457 base pairs, and is predicted to have 3,052 protein-doing genes.

In our previous work, we used an array of bioinformatics tools (*e.g.*, NCBI BLAST, T-COFFEE, WebLogo, TMHMM, SignalP, PFAM, TIGRFAM, *etc*.) to study the genes in *M. ruber* predicted to be involved in proline biosynthesis. We are proposing that proB and proA in *M. ruber* also exists as an operon (Figure 3). Noticeably absent, however, is a putative Shine-Dalgarno sequence positioned 8-13 bases from the start codon of the proA gene.

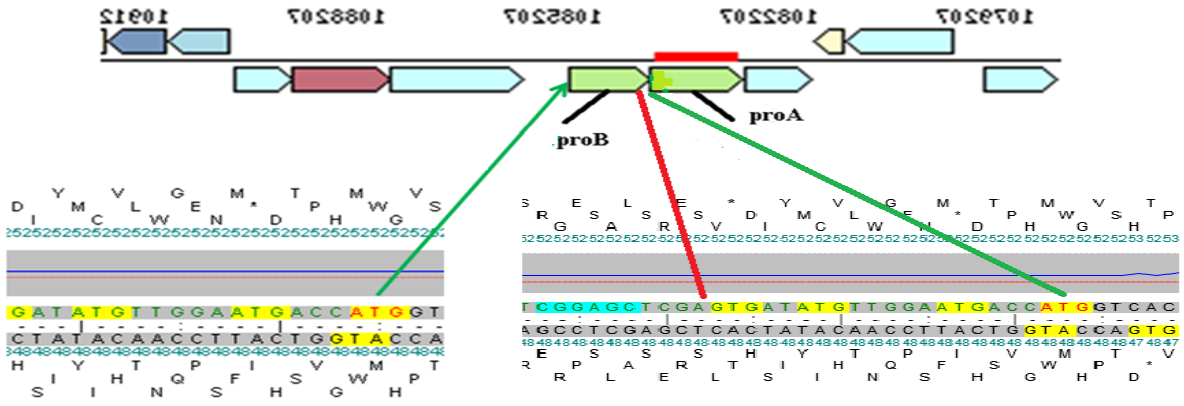

Figure 3. Chromosome region containing the putative proBA operon in *M. ruber*. Top panel: broad arrows identify putative ORFs pointing in the direction of transcription; proB and proA are labeled. DNA coordinates are backwards because the proB and proA genes are on the complementary DNA strand, as compared to the same region in *E. coli* K12 MG1655. Vertical arrows identify the proB start and stop codons and proA start codon. Both images were taken from IMG/edu.org and accessed through the Gene Details pages for *M. ruber* proB and proA.

In previous work, Dr. Scott's BIOL375/Molecular Genetics students attempted to provide functional evidence confirming the role of putative *M.* ruber proA, proB and proC in proline biosynthesis using the complementation assay described by GENI-Science group (GENI-

Science.org, 2015) Complementation, as described by the project Functional Genomics of Arginine Biosynthesis in *A. tumefaciens* C58 within GENI-Science, studies the ability of genes of interest(s) to restore wild-type function to *E. coli* Keio null strains (Baba et al., 2006). The *M. ruber* and *E. coli* genes are inserted into the pKt1 expression plasmid and then transformed using conventional means into the *E. coli* Keio null strains. In a previous study, it was demonstrated that *M. ruber* genes can be expressed in these Keio strains using pKt1 as the expression vector (Lori Scott, personal communication). In this study, *M. ruber* proA-pKt1 and proC-pKt1 clones complemented proA- and proC- *E. coli* null strains, respectively. However, complementation was not observed for *M. ruber* proB clone or an *M. ruber* proBA clone in an *E. coli* proB- strain, nor did *M ruber* proBA complement an *E. coli* proA- null strain. One possible explanation for the lack of complementation of the *M. ruber* proB-pKt1 clone in the *E. coli* proB- null strain is the necessity of producing an interspecies GK-GSD complex *(i.e., M. ruber* proB forming a complex with the *E.coli* proA), which is why we tested an *M. ruber* proBA-pKt1 clone for complementation. A possible explanation for the lack of complementation of the *M. ruber* proBA-pKt1 clone in the *E. coli* proA- or proB- null stains could be the lack of a recognizable Shine-Dalgarno sequence upstream of the *M. ruber* proA gene.

In this study, we investigate the Shine-Dalgarno issue by inserting an *E. coli* Shine-Dalgarno sequence upstream of the *M. ruber* proA in an *M. ruber* proBA-pKt1 clone using site-directed mutagenesis, followed by repeating the complementation assay. Site-directed mutagenesis involves the alteration of a nucleotide sequence of interest, in our case the intergenic region between *M. ruber p*roB and proA, using synthetically constructed oligonucleotides (Carrigan *et al.*, 2011). In this study will be utilizing site-directed mutagenesis to insert a series of nucleotides that correspond to one of two putative *E. coli* Shine-Dalgarno

sequences. The method of site-directed mutagenesis followed in this study is that of Strategene's QuikChange II XL Site-Directed Mutagenesis Kit. It is a 3-step method: mutant strand synthesis via high fidelity DNA polymerase in PCR, digestion of the template by *Dpn* 1 endonuclease activity, and transformation of mutated DNA into ultracompetent cells (QuikChange II XL Site-Directed Mutagenesis Kit). We hypothesize that the presence of a recognized *E. coli* Shine-Dalgarno sequence in the intergenic region of the M. ruber proBA-pKt1 clone will promote proA expression, and result in complementation of the *M. ruber* proB in an *E. coli* null strain.

Materials and Methods:

Isolation, Purification, and Gel Extraction and Sequencing of *M.* ruber and *E. coli* proBA clones

To confirm the sequence of previously constructed *E. coli* and *M. ruber* proBA-pKt1 clones and the subsequent mutated clones, we sent purified PCR products to the University of Iowa DNA Sequencing facility (Iowa City, IA). -80^{o}C stock cultures were grown overnight in LB/amp (50µg/ml) and then pDNA was isolated using the MidSci plasmid DNA purification kit as described by the supplier. The proBA regions were then amplified using the Promega PCR kit with primers created by IDT (Coralville, IA). The PCR programs used were Promega 3 (annealing temp. of 49˚C) and Promega 4 (annealing temp. of 53˚C) for 30 cycles. The PCR products were then fractionated via gel electrophoresis (1% agarose gel, 100V for 60 minutes) and gel extracted using a MidSci Gel Extraction kit. Table 2 lists the primers used to sequence the clones from both directions to ensure complete coverage.

Table 2. pKt1 forward and reverse DNA sequencing primers

Primer Name	Primer Type	Sequence
Primer 5015	pKT specific Forward primer	5'- TCTGAGGCTCGTCCTGAAT -3'
Primer 5016	pKT specific Reverse primer	5' – TGACGCTTTTTATCGCAACTC -3'

Site-directed mutagenesis

To perform the site-directed mutagenesis and insert the *E. coli* Shine-Dalgarno sequence between *M. ruber* proB and proA, the QuikChange II XL Site-Directed Mutagenesis Kit protocol provided by Strategene, an affiliate of Agilent Technologies, was followed. Two sets of primers (Table 3) were designed using the primer design tools on the Agilent Technologies website. The PCR program utilized in the protocol was Promega 6 at an annealing temperature of 50˚C. Below are the primers used in performing the site-directed mutagenesis.

Table 3. Primers used to insert Shine-Dalgarno site upstream of M. ruber proA in the proBA-Kt1 clone

Primer Name and Sequence	Primer Type	Sequence
Mutant 1 (AGGAGC)	Forward primer	5'GCTCGAGTGATATGTTGGAATGGAGGACCATGGTCACCT
Mutant 1 (AGGAGC)	Reverse primer	5' –AGGTGACCATGGTCCTCCATTCCAACATATCACTCGAG
Mutant 2 (GGAGG)	Forward primer	5'GCTCGAGTGATATGTTGGAATAGGAGCGACCATGGTCACCT
Mutant 2 (GGAGG)	Reverse primer	5'AGGTGACCATGGTCGCTCCTATTCCAACATATCACTCGAGC

Transformation of E. coli and *M. ruber* proBA clones into host *E. coli* proA/proB null strains, and subsequent complementation

To transform the E. coli and *M. ruber* proBA-pKt1 clones into competent *E. coli* Keio null strains, the transformation protocol set of Chung et al. was used (Chung et al. 1989). The protocol for the complementation assay is provided by Dr. Brad Goodner (Hiram College, OH) on his GENI-Science site.

Results:

Figure 4 shows the result of successful amplification of *E. coli* and *M. ruber* proBA. We also see that both *E. coli* and *M. ruber* proBA have the expected number of nucleotides for proBA.

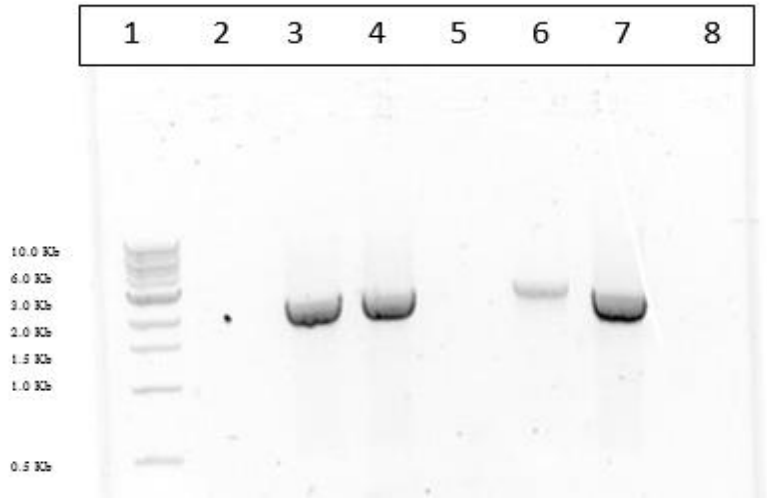

Figure 4. Successful amplification of *E. coli* and *M. ruber* proBA genes. PCR performed by Promega's PCR protocol (Promega 3 program) at 49˚ for 30 cycles and 2X PCR Master Mix; PCR gel purification and extraction performed according to MidSci's gel purification and extraction protocol; gel ran at 100 V for 45 minutes and stained with Sybersafe dye. Lane 1: 10 kb ladder/molecular weight marker; Lanes 3-4: *E. coli* K12MG1655 proB PCR product; Lanes 6-7: *M. ruber* DSM1279 proBA PCR product.

Figure 5 shows the successful amplification of *M. ruber* proBA and *M. ruber* proB from the pkt1 plasmid by PCR. We see successful amplification of *M. ruber* proBA with the expected number of nucleotides for proBA in lanes 3 and 4. However, lanes 6 and 7 does not show evidence of the presence of *M. ruber* proBA, rather it is unknown what exactly the PCR product is. The PCR amplification of this PCR product was most likely the result of sample mix up or improper labeling.

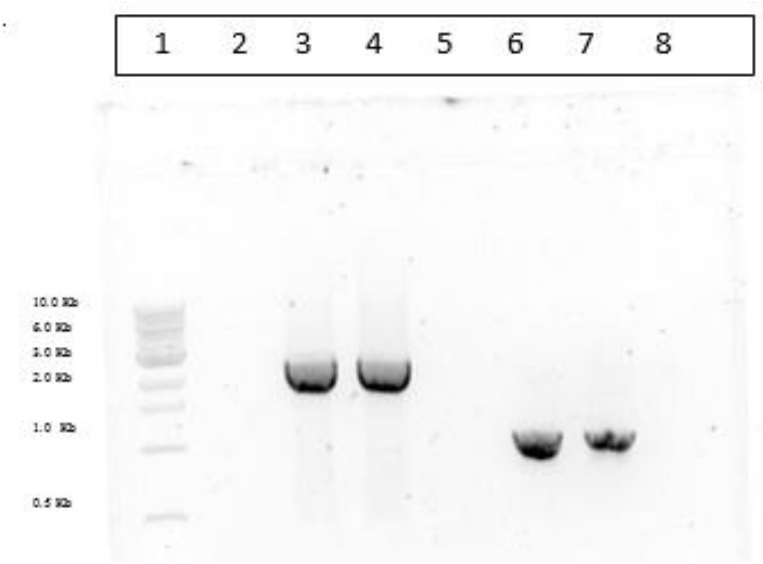

Figure 5. Successful amplification of *M. ruber* proBA and suspected proB genes. PCR performed by Promega's PCR protocol (Promega 4) at 53˚C for 30 cycles and 2X PCR Master Mix; PCR gel purification and extraction performed according to MidSci's gel purification and extraction protocol; gel ran at 100 V for 45 minutes and stained with sybersafe dy*E*. Lane 1: 10 kb ladder/molecular weight marker; Lanes 3-4: *E. coli* K12MG1655 proBA PCR product; Lanes 6-7: Unknown PCR product.

Additionally, it is important to note here that the *M. ruber* clones were sequenced, but the sequencing data is not shown. However, the sequencing data were nBLAST'ed against the *M. ruber* genome and the appropriate hit was obtained. Figure 6 shows the results of a nucleotide

BLAST alignment between *M. ruber* proBA-mutant 1 against the *M. ruber* genome. The hit pulled from the GenBank database shows 99% sequence similarity with *M. ruber* proBA-mutant 1. The discrepancy in the alignment is due to the insertion of the *E. coli* Shine Dalgarno sequence between the *M. ruber* proB and proA genes. This mutation begins at query nucleotide 309. The Shine-Dalgarno insertion in the alignment below is that of –AGGAGC-. This insertion accounts for 5 of the 7 gaps seen in the alignment. Strains that contain this mutant are referred to as *M. ruber* mutant 1 in this study.

Meiothermus ruber DSM 1279, complete genome
Sequence ID: gb|CP001743.1| Length: 3097457 Number of Matches: 1

Range 1: 1084218 to 1084811 GenBank Graphics ▼ Next Match ▲ Previous

Score	Expect	Identities	Gaps	Strand
1062 bits(575)	0.0	593/600(99%)	7/600(1%)	Plus/Minus

Features: gamma-glutamyl phosphate reductase
glutamate 5-kinase

```
Query  10       GGA-GTGGTGGTGGACGCCGGGGCGGCGAAAGCCTTACGCCAGGGCGGGGCCTCGCTCCT  68
               ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084811  GGAGGTGGTGGTGGACGCCGGGGCGGCGAAAGCCTTACGCCAGGGCGGGGCCTCGCTCCT  1084752

Query  69       GCCGGCGGGCATCCTCGAGGTGCGCGGGCAGTTTGGGGTGGGCGAGGCGGTCAGGTGCCT  128
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084751  GCCGGCGGGCATCCTCGAGGTGCGCGGGCAGTTTGGGGTGGGCGAGGCGGTCAGGTGCCT  1084692

Query  129      GGACGAGCAGGGCAACCTGATCGGGGTGGGCCTGGTCAACTACAGCGCCGCCGAGCTCGC  188
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084691  GGACGAGCAGGGCAACCTGATCGGGGTGGGCCTGGTCAACTACAGCGCCGCCGAGCTCGC  1084632

Query  189      CCGCATCAAGCGCCGAAAAACCAGGGAGATCGAGGCCCTGCTGGGCTACAAAAACACCGA  248
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084631  CCGCATCAAGCGCCGAAAAACCAGGGAGATCGAGGCCCTGCTGGGCTACAAAAACACCGA  1084572

Query  249      CGAGGCCATCCACCGCGACTACTTTGCCCTGGCCTCGGAGCTCGAGTGATATGTTGGAAT  308
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084571  CGAGGCCATCCACCGCGACTACTTTGCCCTGGCCTCGGAGCTCGAGTGATATGTTGGAAT  1084512

Query  309      AGGAGCGACCATGGTCACCTCTCCCGAACTCAAGGCATACGCCCAGGCGGCCAGGGCTGC  368
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084511  ------GACCATGGTCACCTCTCCCGAACTCAAGGCATACGCCCAGGCGGCCAGGGCTGC  1084458

Query  369      CGCGCGGGCTTTGTCGACGGCCTCGCCCAGGGCCAAGAACACCGCACTTTTGGCTATAGC  428
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084457  CGCGCGGGCTTTGTCGACGGCCTCGCCCAGGGCCAAGAACACCGCACTTTTGGCTATAGC  1084398

Query  429      AGCGAAGCTCGAGGCCCAGCAAGAGGCCCTTTTTGCCGCCAACCGCCAGGACCTCGAGGC  488
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084397  AGCGAAGCTCGAGGCCCAGCAAGAGGCCCTTTTTGCCGCCAACCGCCAGGACCTCGAGGC  1084338

Query  489      CGCCCAGGCCGCGGGCCTCTCCAAGGCCAAGCTCGACCGGCTGCGACTGGACGAAAAGGT  548
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Query  549      GCTGCGCGACCTGCGAACCGGCCTGCAGCAGGTCGCCGAGATGCCCGACCCCGTCGGCGA  608
               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1084277  GCTGCGCGACCTGCGAACCGGCCTGCAGCAGGTCGCCGAGATGCCCGACCCCGTCGGCGA  1084218
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Figure 6. Nucleotide BLAST comparison of *M. ruber* proBA-mutant 1 against the M. ruber genome, indicating successful insertion of the *E. coli* Shine-Dalgarno sequence (AGGAGC) between *M. ruber* proB and proA. Comparison of the mutant and native sequences was completed utilizing NCBI's nucleotide BLAST.

Figure 7 shows the nucleotide BLAST alignment between the *M. ruber* proBA-mutant 2and the and the *M. ruber* genome. The alignment shows 99% identification of the mutagenic *M. ruber* proBA with the native *M. ruber* proBA. The discrepancy in the alignment is due to the insertion of the *E. coli* Shine Dalgarno sequence between the *M. ruber* proB and proA genes. This mutation begins at query nucleotide 310. The Shine-Dalgarno insertion in the alignment below is that of –GGAGG-. The insertion, however, only accounts for four gaps instead of five, as one of the guanine nucleotides incidentally aligned with the native *M. ruber* proBA strand. Strains that contain this mutant are referred to as *M. ruber* mutant 2 in this study.

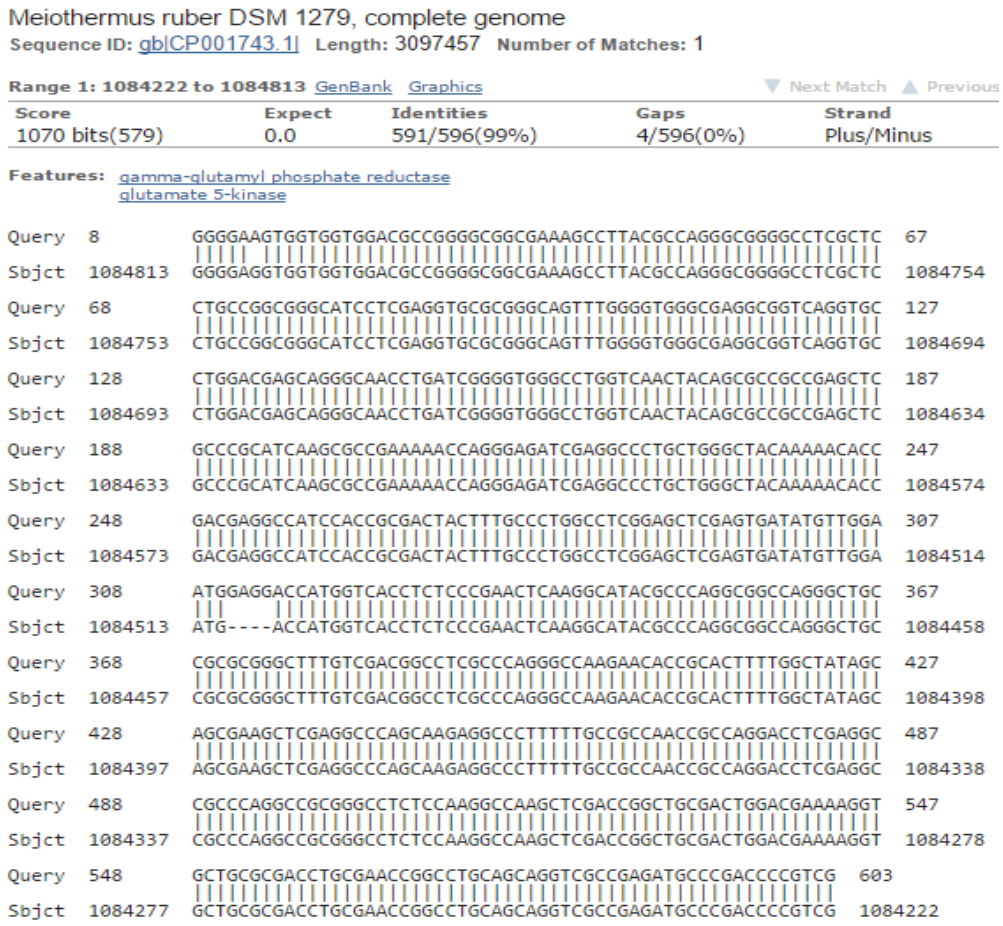

Figure 7. Nucleotide BLAST search of native *M. ruber* proBA against the sequenced site-directed mutagenesis mutant *M. ruber* proBA indicating successful insertion of the *E. coli* Shine-Dalgarno sequence (GGAGG) between *M. ruber* proB and proA. Comparison of the mutant and native sequences was completed utilizing NCBI's nucleotide BLAST.

Figure 8 shows the successful gel electrophoresis results of the isolation, amplification, purification, and eventual extraction of the mutagenic *M. ruber* proBA gene. This gel preceded the actual sequencing of the DNA fragment shown in the BLAST results above, but gave an indication that we had a DNA fragment of the correct size. Lane 4 was not utilized as the quantity of PCR product was not sufficient. The initials in parentheses (LS or MM) indicate the individual who created the mutant.

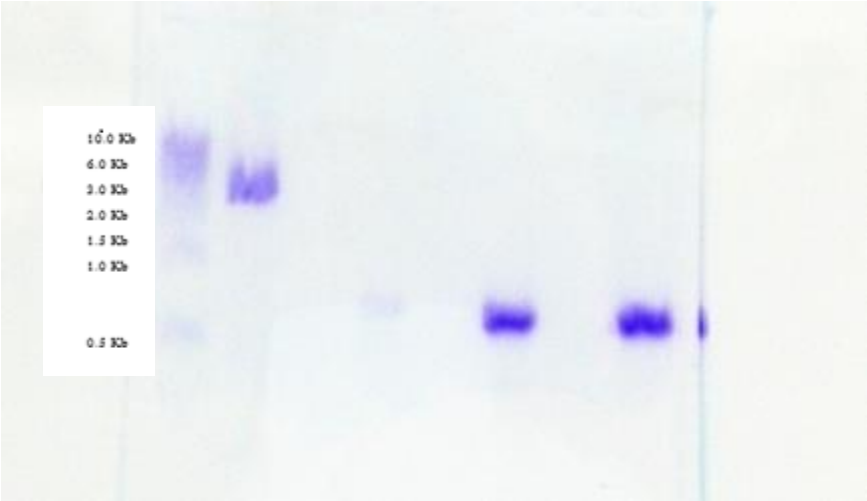

Figure 8. Successful amplification of mutagenic *M. ruber* proBA gene. PCR performed by Promega's PCR protocol and 2X PCR Master Mix; PCR reaction used primer (B.P. 808-1432) at a 1:10 dilution unless otherwise noted. PCR gel purification and extraction performed according to MidSci's gel purification and extraction protocol; gel ran at 100 V for 45 minutes and stained with sybersafe dy*E*. Lane 4 was not used due to low quantity. Lane 1: 10 kb ladder/molecular weight marker; Lanes 2: Mut 2-1 with pkt (1:10 dilution) primers (MM); Lane 4: Mut 2-2 (MM); Lanes 6: Mut 2-4 (MM); Lane 8: Mut 2-3 (MM).

Figure 9 shows the successful gel electrophoresis results of the isolation, amplification, purification, and eventual extraction of the mutagenic *M. ruber* proBA gen*E*. The above gel, again, preceded the actual sequencing of the DNA fragment shown in the BLAST results above,

but gave an indication that we had a DNA fragment of the correct size. As in the first figure, the initials in parentheses (LS or MM) indicate the individual who created the mutant.

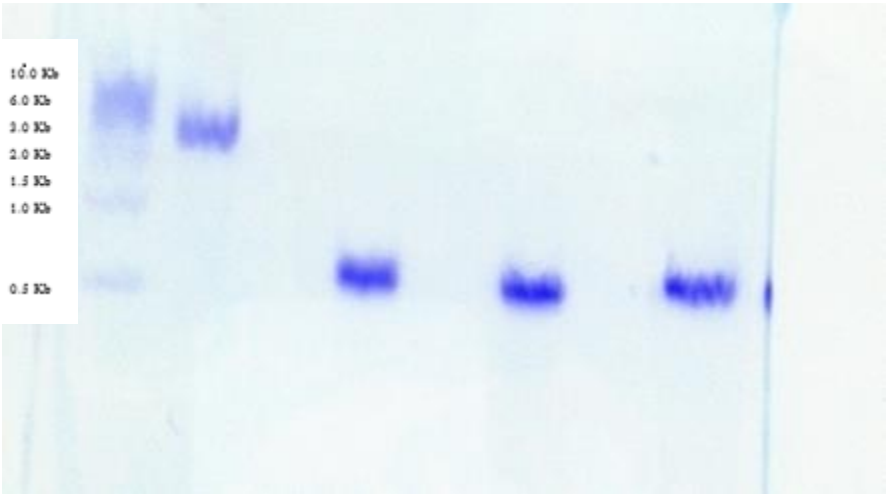

Figure 9. Successful amplification of mutagenic *M. ruber* proBA gene. PCR performed by Promega's PCR protocol and 2X PCR Master Mix; PCR reaction used primer (B.P. 808-1432) at a 1:10 dilution unless otherwise noted. PCR gel purification and extraction performed according to MidSci's gel purification and extraction protocol; gel ran at 100 V for 45 minutes and stained with sybersafe dy*E*. Lane 1: 10 kb ladder/molecular weight marker; Lanes 2: LS Run 2 M1-a with pkt (1:10 dilution) primers (LS); Lane 4: LS Run 2 M1-a (LS); Lanes 6: LS Run 2 M1-b (LS); Lane 8: LS Run 2 M2-a (LS).

Figure 10 shows the successful complementation of *E. coli* null strain for proA with the non-mutagenic *E. coli* and *M. ruber* proBA and with both of the mutagenic *M. ruber* proBA with respective *E. coli* Shine-Dalarno sequences. We also ran an assays of wild type *E. coli* as a positive control to ensure proper and non-lethal growing condition, an *E. coli* null strain for proA as a negative control to ensure the strain was indeed null for the intended protein, and an *E. coli* null strain for proA + base pkt1 to ensure proper plasmid function (no abnormal interactions with the null strain). Expected and desired results were obtained as complementation occurred

between the mutagenic *M. ruber* proBA clones with the Shine-Dalgarno insert and the *E. coli* null strain for proA. However, we also obtained unexpected results as complementation also occurred between the non-mutagenic *M. ruber* proBA and the *E. coli* null strain for proA.

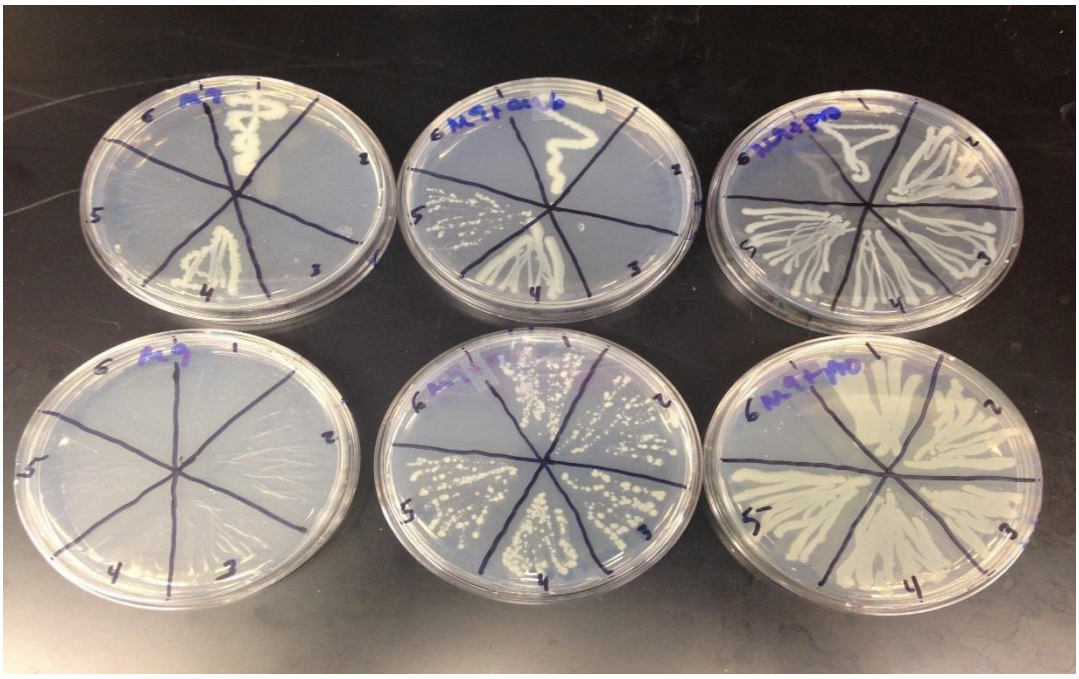

Figure 10. Successful complementation of mutagenic *M. ruber* proBA (with *E. coli* Shine-Dalgarno sequences) and with native *M. ruber*. Top Left plate: Colony growth seen for wild type *E. coli* K12MG1655 and *E. coli* proBA; Top Middle plate: growth seen for wild type *E. coli* K12MG1655, *E. coli* proBA, and *M. ruber* proBA; Top Right plate: growth seen for wild type *E. coli* K12MG1655, null *E. coli* proA strain, null *E. coli* proA + pkt1, *E. coli* proBA; and *M. ruber* proBA. Bottom Left plate: no growth for any of the *M. ruber* mutants; Bottom Middle plate: growth seen for each *M. ruber* mutants (both Shine-Dalgarno mutations). Bottom Right plate: growth seen for each *M. ruber* mutants (both Shine-Dalgarno mutations). The plates on the left contained minimal media (nutrients); the plates in the middle contained minimal + arabinose media (nutrients); the plates on the right contained minimal + proline media.

Discussion and Conclusion:

In analyzing and interpreting the results of the present study, we arrive at some interesting findings and conclusions. The most interesting of the results obtained is found within the complementation assays. However, let us establish an understanding of the results that serve as a premise to these results themselves. Looking at the initial isolation, amplification, purification, extraction, and sequencing of the non-mutagenic proBA from *M. ruber*, and *E. coli* at that, we see result that we would expect and that confirm the presence of the *M. ruber* proBA non-mutagenic clone. The gels indicate that the isolated DNA fragments are of the right kilo base length and the right molecular weight of *M. ruber* (and *E. coli*, but *M. ruber* is the organism of interest here) non-mutagenic *M. ruber* proBA. Additionally, the sequencing data of these clones, even though not explicitly shown in the results, provided the means to identify the DNA fragments isolated were indeed *M. ruber* proBA. The site-directed mutagenesis insertion of an *E. coli* Shine-Dalgarno sequence between *M. ruber* pro B and proA also returned expected and desired results. The gel electrophoresis analyses indicate that a fragment the size of the proBA operon had been isolated. Analyzing the nucleotide BLAST alignment of the sequenced mutagenic *M. ruber* proBA and the non-mutagenic *M. ruber* proBA and the relative position of the insertion of the Shine-Delgarno sequence in reference to figure 3 in the introduction, we can confidently conclude that the site-directed mutagenesis was successful in introducing the *E. coli* Shine-Dalgarno sequence in the intergenic region of *M. ruber* proBA.

However, we now turn to the peculiar, but interesting results. In analyzing the complementation assays, it is indicated that the positive control of the wild type *E. coli* grew on all three media as expected, thus indicating that the media was nutritious and non-lethal to promote growth. Furthermore, the negative controls of the *E. coli* null strain for proA and the *E.*

coli null strain for proA + functional base pkt1 plasmid only grew on the minimal + proline media, indicating that the null strain even in the presence of a functioning pkt1 plasmid could only grow in the presence of environmental proline. However, when we analyze the complementation assays of the non-mutagenic *M. ruber* proBA with the *E. coli* null strain from proA, the same complementation assay that appeared to be unsuccessful in our previous study, we see an indication of growth and successful complementation. In the analysis of the complementation between the mutagenic *M. ruber* proBA and the *E. coli* null strain for proA, we see the desired and expected result of successful growth and complementation. In comparison to the mutagenic *M. ruber* proBA complementation with the *E. coli* null strain for proA, the complementation of non-mutagenic *M. ruber* proBA resulted in significantly less growth in the complementation. From these results the hypothesis that inserting an *E. coli* Shine-Dalgarno sequence in the intergenic region of *M. ruber* proBA would facilitate and promote growth and complementation in an *E. coli* host null strain for proA is inconclusiv*E.* It is unclear as to why complementation of non-mutagenic *M. ruber* proB A with the *E. coli* null strains for proA seemed to result in growth in this study and not in the our previous study. One possibility might be due to the setting and the conditions that preliminary study was performed in. The preliminary study was performed by a larger group contributors in a less sterile environment (a classroom used by many people). Additionally, with a larger number of contributors, there is more room for procedural error, improper or unsterile technique, cross-contamination, and even general differences in procedural work. Moreover, although, a large concern in the discrepancy of the results between the preliminary study and this study is the factor of the quality and functional ability of several of the materials used such as media, plasmids, host cells, primers, etc. that may have differed between the preliminary study and this present study.

Another interesting finding in the complementation assays was the apparent increased growth of the mutagenic *M. ruber* proBA clones in complementation with the *E. coli* null strain for proA compared to that of the growth of the non-mutagenic *M. ruber* proBA clones in complementation with the *E. coli* null strain for proA. This then begs the question of: did the insertion of the *E. coli* Shine-Dalagarno sequence facilitate and promote greater growth and complementation of *M. ruber* proBA to the *E. coli* null strain for proA? The possibility of this occurrence is surely an important consideration in progressing with further studies in the study of the biosynthesis of proline in *M. ruber*. One possibly detrimental complication and possible explanation for this increased growth, however, is the question of the number of cells that were placed and/or survived in each complementation assay. This may be a facet on which this experiment could be improved upon. If the amount of cells could be controlled, possibly through concentration or volume, this may produce more reliable results. One direction that future studies might take in answering this question is to use varying degrees of nutrient concentrations (namely arabinose which allows for the expression of the pkt1 plasmid) in the media and determine whether the insertion of the *E. coli* Shine-Dalgarno sequence in the *M. ruber* proBA operon has a prominent effect in facilitating, promoting, and inducing increased complementation and growth in an *E. coli* null strain for proA.

While this study ultimately resulted in inconclusive results for the proposed hypothesis, it did produce some exciting, progressive, and thought provoking results. With the indication that native non-mutagenic *M. ruber* proBA can complement, although what appears to be a lesser degree than that of the mutagenic *M. ruber* proBA, an *E. coli* null strain for proA, future studies might look in two directions. One, which we have already mentioned would be to investigate the degree of strength between the complementation and growth of the non-mutagenic and

mutagenic *M. ruber* proBA with the *E. coli* null strain for proA. The second direction, especially in light of successful complementation of the native non-mutagenic *M. ruber* proBA with the *E. coli* null strain, would be the study of the exact interspecies relationships and compatibilities be between each of the *M. ruber* proline genes and the operon as a whole.

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