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Cloning, Sequencing, and Characterization of *Luciola italica* Luciferase

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Presents an Honors Thesis

under the supervision of Dr. Bruce R. Branchini

April, 2006

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Bruce R. Branchini for introducing me to this project and advising me through it.

I would also like to thank everyone in the lab including Tara Southworth, Danielle Ablamsky, and Martha Murtiashaw for all their help throughout this project; I would have not been accomplish this without them.

I want to thank Evelyn Bamford and Maureen Ronau for little things and words of encouragement, which were such a big help in dealing with this difficult year.

Lastly, I want to thank Dr. David Cullen and Daniel Odom for being great and helpful readers for this thesis.
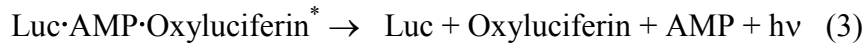
ABSTRACT

The characteristic yellow-green light of a firefly is the result of a multi-step reaction catalyzed by the luciferase enzyme. This enzyme has many applications in the biomedical field and ongoing work is being done to alter its properties to better fit these applications. The purpose of this project was to clone the *Luciola italica* luciferase cDNA and to express, purify and fully characterize the corresponding bioluminescence-catalyzing enzyme in hopes of obtaining novel bioluminescent materials. Fireflies were collected in the countryside of Bologna, Italy, flash frozen in liquid nitrogen and total RNA was extracted from the firefly lanterns. The *L. italica* luciferase cDNA was successfully cloned by RT-PCR using a gene-specific primer set based on the DNA sequence of the Eastern European *Luciola mingrelica* luciferase gene. The *L. italica* cDNA was determined to be 1647 base pairs in length with an open reading frame of 548 amino acids. Initial characterization of the enzyme showed that the *L. italica* protein exhibits bioluminescent activity similar in intensity to the common North American *Photinus pyralis* luciferase; however it produces light that is slightly red-shifted (having maximum emission at 564 nm). By steady state kinetics analysis, the *L. italica* K_m for LH_2 was found to be 0.095 mM, and that of *P. pyralis* is 0.015 mM. On the converse, both enzymes had similar K_m values for Mg-ATP (0.160 mM for *P. pyralis* and 0.180 mM for *L. italica*). The *L. italica* enzyme was found to sustain its light in the visible region for a longer period of time than the *P. pyralis* enzyme. Phylogenetic analysis showed that the *L. italica* luciferase gene has 95.8% and 95.6% amino acid sequence identity to the *Hotaria unmunsana* (Korea) and *Hotaria parvula* (Japan) luciferase proteins, respectively. The processes that were used to clone the *Luciola italica* luciferase gene, characterize the protein, and optimize protein growth conditions are presented in this study.

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INTRODUCTION

Firefly luciferase is a ~62 kDa oxygenase enzyme most commonly used from the North American firefly *Photinus pyralis* [1, 2]. The enzyme catalyzes a multi-step reaction that requires two substrates: Luciferin (LH_2) and Mg-ATP. The first involves the adenylation of LH_2 with Mg-ATP to form an acyl-adenylate intermediate (LH_2-AMP) and pyrophosphate (eq. 1). This intermediate then undergoes an oxidation reaction forming CO_2 and the excited product oxyluciferin (eq. 2). The characteristic yellow-green light seen ($\lambda_{max} \approx 560nm$) is given off (eq. 3) when oxyluciferin returns to the ground state [1, 3]. The firefly uses this enzyme-catalyzed reaction to emit a yellow-green light for the purpose of attracting a mate.

$$\text{Luciferase (Luc)} + LH_2 + Mg^{2+}\text{-ATP} \leftrightarrows \text{Luc·}LH_2\text{-AMP} + Mg^{2+}\text{-PP}_i \quad (1)$$

$$\text{Luc·}LH_2\text{-AMP} + O_2 \rightarrow \text{Luc·AMP·Oxyluciferin}^* + CO_2 \quad (2)$$

$$\text{Luc·AMP·Oxyluciferin}^* \rightarrow \text{Luc + Oxyluciferin + AMP} + h\nu \quad (3)$$

This reaction has certain qualities such as sensitivity, rapidity, and the non-invasive method of quantification [4] that allow for efficient applications in a wide variety of biochemical experiments. It is a particularly useful as a tool in the ultra-sensitive detection of ATP, a reporter gene for monitoring transcriptional activity, a biosensor for chemical toxins, a probe for monitoring protein folding *in vivo*, and a probe for the actions of local anesthetics [1]. In the medical field the luciferase reaction is applied in cancer [5] and AIDS [6] studies, and evaluating the *in vitro* activities of drugs [7]. Luciferase is also used for real-time expression in live animals. It has already been

expressed (Figure 1) in tobacco and carrot plants, mammalian cells, zebra fish, and *Drosophila* [8].

Yet another application of the luciferase bioluminescence reaction is for dual reporter assays, which is involves in the quantitation of gene expression using two reporter luciferases. Mutagenesis work has been done to alter the spectral properties of luciferase to change its light emission color to red or green [9, 10]. These studies are being taken further by using random mutagenesis to create brighter and more thermostable spectrally shifted mutants.

Figure 1. Plant Expressing Firefly Luciferase (Kiwipedia.com).

The luciferase gene of the North American firefly *Photinus pyralis* was first identified by DeLuca [11] and has since been the most widely used in bioluminescence studies. A total of 17 other luciferase genes have also been reported, including *Luciola*

cruciata [12], *Luciola lateralis* [13], the European firefly *Luciola mingrelica* [14], *Hotaria unmunsana* [15] and *Hotaria parvula* [16] from the Luciolinae family, and the European glow-worm *Lampyris noctiluca* [17]. There are only two fireflies reported to exist in Italy; one is *Luciola lusitanica* [18, 19], about which little is known, and the other, more predominant *Luciola italica* (Figure 2), which is the subject of this thesis.

Figure 2. Images of live *L. italica* fireflies (ventral and dorsal views).

One purpose of this thesis is to report the cloning and sequencing of the *L. italica* luciferase gene in order to identify novel bioluminescent materials for study. Amino acid comparisons among firefly species can be used to determine important structure-function similarities and differences among their luciferase enzymes. Amino acids shared between species' sequences are likely to serve an important role in the catalysis of this reaction and identifying them will lead to a better understanding of the biochemical properties of luciferase, including the basis for the bioluminescence color. Another purpose is to identify the properties of *L. italica* luciferase so it can be used it in mutagenesis studies.

MATERIALS AND METHODS

Materials. The following items were obtained from the indicated sources: Mg-ATP-bacterial source (Sigma); XL 10-Gold ultra competent cells (Stratagene); Glutathione Speharose 4B and pGEX-6P-2 expression vector (Amersham Biosciences); oligonucleotides and Platinum® *Pfx* DNA polymerase (Invitrogen); restriction endonucleases and T4 DNA ligase (New England Biolabs). D-Firefly luciferin was a generous gift from Promega.

General methods. Light measurements were made in 8 x 50 mm polypropylene tubes (Evergreen Scientific, Los Angeles, CA) placed in the sample compartment of either an SLM-Aminco Chem Glow II or a Turner TD-20e luminometer interfaced to a Strawberry Tree Inc. (STI) A/D converter (with a 0.05-0.10 s sampling rate) and stored to a Macintosh SE computer. Light measurements were quantified with customized versions of the STI Workbench software. All measurements were corrected for the spectral response of the Hamamatsu 931B photomultiplier tube. All luciferases in pGEX-6P-2 plasmids were expressed in *Escherichia coli (E. coli)* strains BL21 or XL10Gold at 22 ºC.

Cloning and Sequencing

Specimen collection. The firefly *Luciola italica* (Figure 2) was collected from Bologna-Paderno, Bologna Villa Ghigi Park and Bologna, Eremo di Tizzano, Italy. The collected fireflies (233 total) were transferred to the laboratory where they were flash frozen while alive in liquid nitrogen, counted, and stored at −80 °C overnight.

Total RNA extraction. Fireflies were transferred to a liquid nitrogen filled dewer, picked out individually, and their lanterns were removed. Before transfer to a pre-frozen mortar, they were allowed to sit for 5 min in liquid nitrogen. While repeatedly adding liquid nitrogen, the lanterns were ground to a powder using a pre-frozen mortar pestle. After the liquid nitrogen evaporated, the dry powder was transferred to a 1.5 mL eppendorf tube and RNA was extracted using the RNeasy® mini kit (Qiagen) and was eluted in RNase-free water. The concentrations were determined by UV absorption spectroscopy at 260 nm.

RT-PCR of Luciola italica *luciferase gene.*   The first strand of cDNA was synthesized at 50 °C for 50 min at 85 °C for 5 min with ~5 µg RNA and Oligo(dT)$_{20}$ primers using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The following primers were designed based on the luciferase cDNA sequence of the sources indicated and were used in attempts to carry out amplification of *L. italica* cDNA : *Luciola mingrelica*, 5'-GTC CCT AAA CGG TAG AGG AAA AG-3' ; *Lampyris noctiluca*, 5'- GAG ACA CTA ACG CGC TAA TAT C-3'; *Luciola cruciata*, 5'- CAA GTA CGG TTT CAA AGT GA-3'; 350 bp and 1600 bp internal primers (based on sequence alignments of species in the Luciolinae subfamily), 5'- GG(A,G, or T) GTA GGT GTT GCT CCA AC(A or T) AAT G-3' and 5'- CCA CAA A(A or C)C GAA C (T, G, A, or C)C CAC CAC G(C or T)C AAC G-3' respectively; based on alignments of *Pyrocoelia miyako, Lampyris noctiluca, Photinus pyralis,* and *Pyrocoelia rufa* sequences, 5'- ATG GAA GAT GAT AGT AAA CAT ATT-3', and 5'- ATG GAA GAT GCA AAA CAT ATT-3'; based on alignments of *Luciola mingrelica*, *Hotaria parvula, Hotaria*

unmunsana, Luciola cruciata, and *Luciola lateralis* sequences*,* 5'- T GTG AAA ATG

GAA ATG GGA-3', and 5'- T GGA ACA ATG GAG AAC GA-3'. The successful PCR

amplification of cDNA was carried out with the primers based on *Luciola mingrelica*

luciferase cDNA under the following conditions: initial denaturation at 94 °C for 5 min, a

35-cycle amplification (94 °C for 30 s, 52 °C for 45 s, and 68 °C for 1.5 min), and a final

extension at 68 °C for 5 min. The PCR products were purified using the QIAquick® PCR

purification kit (Qiagen), and eluted in sterile water. Purified products were verified by

1.0% agarose gel electrophoresis. DNA sequencing of the PCR-amplified cDNA was

performed with a capillary array sequencer CEQ2000XL system (Beckman Coulter Inc.,

Fullerton, CA) at the University of Bologna and at the W.M. Keck Biotechnology

Laboratory at Yale University.

Insertion of cDNA into pGEX-6P-2 plasmid. The primers 5'-TTT AAT CCC

GGG GTC CCT AAA CGG TAG A-3' and 5'-CTA AGC CTC GAG TCT TCT GAG

TAG TT-3' were used to introduce SmaI and XhoI restriction sites, respectively (an

underline represents the endonuclease recognition sites). PCR amplification and

purification of the PCR products were performed as previously stated. The cDNA

fragments were digested (1 hr at 37 ºC) by XhoI and SmaI and ligated (16 hrs at

16 ºC) into the pGEX-6P-2 plasmid, which was also digested with SmaI and XhoI. The

ligated products were then gel purified using the QIAquick® Gel Extraction Kit (Qiagen),

transformed into *E. coli* XL-10 Gold ultra competent cells, and plated on Luria-Bertani

plates containing ampicillin (100 µg/mL). Positive bioluminescence was observed and

colonies were picked at random. Plasmid DNA was purified using the Perfect Prep®

Plasmid Mini kit (Invitrogen) and analyzed by 1% agarose gel electrophoresis. Plasmids of the expected size (containing the *L. italica* cDNA) were sent for sequencing to the W.M. Keck Biotechnology Laboratory at Yale University. One plasmid contained the entire coding sequence but it was determined to be out of reading frame because the particular colony that was picked was evidently dark. We chose to re-align this sequence in the pGEX-6P-2 plasmid.

Alignment of the cDNA reading frame for protein expression. The QuickChange[®] Site-Directed Mutagenesis kit (Stratagene) was used to correct the reading frame of the *L. italica* luciferase cDNA in the pGEX-6P-2 plasmid. The primer 5'-GA TTC TCA CAC GCT AAG GAC CCA ATT TAC GGA AAC CAA GTT TC-3' and its reverse complement were first used to remove a BamHI restriction endonuclease site within the cDNA sequence (underline represents a silent mutation to remove site). The primer 5'-CG GTA GAG GAA AAG TTT GGA TCC **ATG** GAA ACG GAA AGG GAG G-3' and its respective reverse complement were then used to introduce a BamHI site immediately preceding the start codon of the cDNA sequence (underline represents restriction site and bold represents start codon). Products were digested with BamHI and XhoI, purified from an agarose gel as previously described, and ligated into the pGEX-6P-2 plasmid (which was also digested with BamHI and XhoI). The re-aligned *L. italica* luciferase cDNA was sequenced at the W.M. Keck Biotechnology Laboratory at Yale University.

Expression of L. italica *Luciferase as a GST-fusion protein in bacterial colonies.*
BL-21 *E. coli* cells were transformed with plasmids containing *L. italica* luciferase cDNA
and plated on nitrocellulose filters in Luria-Bertani plates containing ampicillin (100
μg/mL). They were then screened for bioluminescence as previously described.
Colonies that displayed positive bioluminescence were selected and plasmid DNA was
purified with the GenElute™ Plasmid Miniprep kit (Sigma).

DNA sequencing and GenBank accession numbers. The cDNA sequence of
Phengodes sp. luciferase was obtained from Keith V. Wood (personal communication)
and the sequence of *Pyrearinus termitilluminans* was determined by Viviana *et al.* [20].
The accession numbers of the sequences obtained from GenBank® are: *Luciola italica*
(this study), **AY633557**, *Cratomorphus distinctus*; **L39929**, *Hotaria parvula*; **AF420006**,
Hotaria unmunsana; **X89479**, *Lampyris noctiluca*; **AY742225**, *Lampyris turkestanicus*;
M26194, *Luciola cruciata*; **DQ138966**, *Luciola italica*; **U51019**, *Luciola lateralis*;
S61961, *Luciola mingrelica*; **M15077**, *Photinus pyralis*; **D25415**, *Photuris pennsylvanica*;
D25416, *Photuris pennsylvanica*; **U31240**, *Photuris pennsylvanica*; **AF139645**,
Phrixothrix hirtus; **AF139644**, *Phrixothrix vivianii*; **L39928**, *Pyrocoelia miyako*;
AF328553, *Pyrocoelia rufa*; **Q7M4K3** (**S29352)**, *Pyrophorus plagiophthalamus* (Green);
S29353, *Pyrophorus plagiophthalamus* (Yellow-Green); **Q7M4K2** (**S29354)**,
Pyrophorus plagiophthalamus (Yellow); **QM4K1 (S29355)**, *Pyrophorus
plagiophthalamus* (Orange).

Protein Expression and Purification

Protein expression. Starter cultures (5 mL) of BL21 *E. coli* cells (containing the pGEX plasmid with *L. italica* luciferase cDNA) were grown in Luria-Bertani broth containing 100 μg/mL ampicillin (LB+amp.) at 37 ºC with shaking at 325 rpm overnight. This was diluted (1:100) into 250 mL LB+ amp in a 1 L flask and the bacteria were allowed to grow until their mid-log phase (OD_{600}= 0.4-0.6). They were moved to a 22 ºC or 18 ºC shaker and allowed to equilibrate for 10 min before induction with 0.1 mM IPTG and were grown overnight at 22 ºC or 18 ºC with shaking at 325 rpm. The bacterial cultures were pelleted by centrifugation (12,000 rpm) at 4 ºC for 1 min and stored at -80 ºC.

Protein purification. Bacterial pellets were resuspended in 25 mL resuspension buffer (150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 [pH 7.3] containing 0.5 mM dithiothreitol [DTT] and 0.1 mM phenylmethanesulfonyl fluoride [PMSF]) until the suspension was uniform. Cells were allowed to sit for 20 min on ice after addition of lysozyme (1 mg/mL) and then sonicated for 30 s in 10 s bursts. The lysate was treated with DNase (5 μg/mL) and RNase (10 μg/mL), allowed to sit for 10 min on ice, and then Triton X-100 was added (to a final concentration of 1%). The unwanted cellular material was pelleted by centrifugation at 13000 rpm for 45 min. The luciferase GST-fusion proteins were separated from other proteins using Glutathione Sepharose® (GST) 4B affinity chromatography according to the manufacturer's instructions. The purified luciferase proteins were cleaved from the GST tags by incubation with PreScission protease in cleavage buffer (CB: 50 mM Tris-HCl [pH 7.0],

150 mM NaCl, 1 mM EDTA, 1 mM DTT) overnight at 4 ºC. Protein fractions of ~2 mL were eluted with CB and stored at 4 ºC with 2% glycerol and 0.8 M ammonium sulfate.

Small scale expression and partial purification for optimization of luciferase growth conditions. Starter cultures of 5 mL were made as previously described and diluted (1:100) into 100 mL LB+amp in 1 L flasks. The cultures were allowed to grow to an OD_{600} of ~0.5 and transferred to an 18 ºC or 22 ºC incubator to equilibrate for 10 min. The expression of luciferase protein was induced with 0.1 mM and the cultures were grown overnight. Various time points were taken by removing 5 mL of culture; cells were pelleted by centrifugation at 5,500 rpm for 5 min, and pellets were stored at −80 ºC.

The purification procedure was followed as previously stated but solutions were scaled down for a 5 mL culture up to the point of adding 20% Triton X-100. The cultures were then centrifuged at 2500 rpm at 4 ºC for 15 min and 500 μL of the supernatant was transferred to pre-frozen eppendorf tubes. These were centrifuged at 14,000 rpm for 5 min at 4 ºC and activity of the crude lysates was measured under saturating (270 μM LH_2) and non-saturating (70 μM LH_2) conditions. Relative activity units (U/5 mL) were plotted vs. induction time (hours).

Characterization

Standard assay reagents. LH$_2$ was prepared by dissolving D-Luciferin into 1 mL glycylglycine (gly-gly) buffer (25 mM pH 7.8) and vortexing. The concentration was determined (for all activity assays) by measuring the absorbency of a 1:40 dilution of the LH$_2$ solution at 266 nm (extinction coefficient at 266 nm= 7600).

Mg-ATP (6 mg/mL) was prepared by dissolving 0.06 g Mg-ATP into 9 mL of gly-gly buffer. The pH was adjusted to between 7.8 and 8.0 and the volume was brought up to 10 mL with gly-gly buffer. The concentration of Mg-ATP was determined (for the Mg-ATP K_m assay only) by measuring the absorbance of a 1:100 dilution at 259 nm (extinction coefficient at 259 nm= 1.6×10^4).

Measuring specific activity. Before measuring activities of proteins, a standard assay was performed for *P. pyralis* wild type protein with 2 µL of a 1:5 dilution in CBA (CB + 0.8 M NH$_4$SO$_4$ + 2% glycerol), 400 µL of 91 mM LH$_2$ (final concentration of 70 µM), 120 µL Mg-ATP at 350 V/1X gain (Turner TD-20e luminometer) or 2 V/ 1X gain (custom light box with R 928 PMT). The specific activity was calculated using the formula:

$$\text{Specific Activity} = \frac{\begin{array}{c}\text{average}\\ \text{peak height} \times 1.2\end{array}}{\text{milliliters of enzyme used} \times \dfrac{\text{enzyme concentration}}{\text{dilution factor}}}$$

This value obtained was compared to the standard value for the *P. pyralis* protein (135,000 units/mg protein) to make sure the luminometer was working properly. Specific activity for the *L. italica* protein was measured using 2 µL enzyme (diluted as necessary), 400 µL 360 µM LH$_2$ (final concentration 270 µM), and 120 µL Mg-ATP

(automatically injected). The final specific activity value was determined from averages of different values obtained on different days.

LH₂ steady-state kinetic constant. The procedure as described above was followed for the LH_2 K_m assay while varying the concentrations of LH_2. This is done to determine the concentration of LH_2 at which the enzyme is saturated and is exhibiting the maximum rate of reaction (V_{max}). The K_m is the concentration of substrate at half the V_{max} value. The assays consisted of ~1.8 μg enzyme, 19.2-600 μM LH_2, a constant concentration of Mg-ATP (2 mM final), and 25 mM gly-gly buffer to give a final volume of 522 μL. The light intensities were recorded and plotted vs. concentrations of LH_2 using Enzyme Kinetics Pro 2.34 (SynexChem™). The graph was plotted as the data were obtained until V_{max} was reached (light intensity values level off when saturating concentrations of LH_2 are reached). The K_m and V_{max} for the protein were calculated through the program by using a nonlinear least-squares method and observed as a Michaelis-Menton hyperbola. The k_{cat} value was determined by using the V_{max} to calculate the specific activity in U/mg, converting this to photons/s/mg and finally to the k_{cat} value in s⁻¹.

Mg-ATP steady-state kinetic constant. The procedure was followed as described above but with varying concentrations of Mg-ATP and a constant concentration of LH_2. This was done in order to determine the concentrations at which V_{max} and K_m occur for Mg-ATP. The LH_2 K_m assay was done first because it is necessary for the Mg-ATP assay to be carried out with LH_2 at its saturating concentration. The assays consisted of

~1.8 µg enzyme, 20-2000 µM Mg-ATP, a constant concentration of luciferase (400 µL of 360 µM to a final of 270 µM), and 25 mM gly-gly buffer to give a final volume of 522 µL. The data was plotted and processed as described above.

Bioluminescence spectra. Bioluminescence emission spectra with LH_2 (270 µm final concentration for *L. italica* and 70 µM final concentration for Ppy) and Mg-ATP (6 mg/mL) with various buffers at different pH's (Mes: pH 6.0, gly-gly: pH 7.0 and 7.8, and Tris: pH 8.6) were obtained using a Perkin-Elmer LS55 luminescence spectrometer while in the "bioluminescence" mode. Data were collected over the wavelength range of 480-680 nm in a 1 mL optical glass cuvette. Gate and delay times, detector voltage, scan rate, and slit width were adjusted to optimize the instrument response. Data were corrected for the spectral response of the R928 photomultiplier tube using the Perkin-Elmer FL WinLab software. This correction was used to calculate a more accurate specific activity by incorporating it into the equation.

Rise, decay, and integration. Light intensities were measured over a period of 15 minutes under the same assay conditions previously stated under 'Measuring Specific Activity' using Labview 7.0. Rise time was determined by recording the amount of time it takes to reach the maximum light intensity and decay time by recording the amount of time it takes this maximum intensity to decay 80% (20% intensity left). Integrated specific activity was also calculated using the same program.

Insertion of AGG to Replace AKM (Peroxisome Targeting Site) in *L. italica* cDNA

Site-directed mutagenesis. The primers 5'- AG AAA CCA CAA GCC <u>GGG</u>

<u>GGG</u> TAA ATC GGT CAA AAT G-3' and its reverse complement were used to replace

the codons encoding amino acids lysine and methionine (represented by underlines) by

glycine. This modification removes a BsaBI restriction site. The primer 5'-G GGG GGT

AAA TCG GTC AAA ATT <u>CTA</u> GAC ATG TAA CTA-3'

and its reverse complement were used to introduce an XbaI site in order to sub-clone the

cDNA into a pCBR-basic vector. PCR conditions and procedures were implemented as

previously described.

Expression in bacterial colonies. PCR products were transformed into *E. coli*

cells and the brightest colonies expressing the gene were picked. The plasmid was

isolated from the cells and a restriction digest with BsaBI was performed to verify

insertion of the mutation. Positive colonies were sent to the W.M. Keck Biotechnology

Laboratory for sequencing.

Protein purification and initial enzyme characterization. The *L. italica* protein

containing the AGG mutation was expressed and purified according to the previous

procedure. Specific activity measurements and bioluminescence measurements (pH 7.8)

were done according to the previous method.

RESULTS AND DISCUSSION

Collection of Luciola italica *fireflies.* This project involving the cloning of

L. italica luciferase cDNA had previously been attempted in June 2004 and was not

successful because a sufficient amount of RNA could not be obtained. The first group

had left behind 10 fireflies, which they had stored at -80 ºC for later use. We returned to

Bologna in late May, 2005 and collected fireflies over a period of three days. The first

collection was done by Professor Aldo Roda in a field just outside of Bologna on 5/29/05.

The 22 fireflies obtained from this collection were kept alive until the RNA isolation the

following day. The next two collections took place on 5/30/05 and 5/31/05 beginning

around 10PM in Bologna-Paderno, Bologna Villa Ghigi park and Bologna, Eremo di

Tizzano; 79 and 154 fireflies were collected, respectively. The fireflies collected on the

latter two days were flash frozen in liquid nitrogen immediately upon returning to the lab

and stored at -80 ºC.

Total RNA isolation from Luciola italica *fireflies.* There were six RNA isolations

done and the best yield was obtained in trial 6 (Table 1). RNA from trials 1 and 6 were

used in successful PCR reactions on the day RNA was isolated. If the RNA was stored

for more than a few days however, the PCR reactions it was used for were not successful.

What is isolated during this procedure is the total RNA contained in the lantern cells; it

mostly consisted of ribosomal RNA. We were interested in the mRNA, which is present

in smaller amounts and is easily degraded. The mRNA had apparently degraded over the

period of a few days when stored at –20 °C.

Table 1: *Total RNA Isolations from* L. italica *fireflies (highest yield in bold)*

Trial	Number of tails used (date collected)	Concentration	Elution Volume	Total Yield (ng per tail)
1	22 (1/29/05)**	167 ng/μL	40 μL	6.7 μg (304 ng/tail)
2	10 (6/04)*	108 ng/μL	30 μL	3.2 μg (320 ng/tail)
3	40 (1/30/05)*	50 ng/μL	49 μL	2.45 μg (61 ng/tail)
4	34 (1/30/05)	171 ng/μL	50 μL	8.55 μg (251 ng/tail)
5	50 (1/31/05)	172 ng/μL	60 μL	10.32 μg (256 ng./tail)
6	**16 (1/31/05)****	**600 ng/μL**	**30 μL**	**18.16 μg (1125 ng/tail)**

* Not used in an RT-PCR reaction.
**Used in a successful RT-PCR reaction

RT-PCR of Luciola italica *luciferase gene.* Primers were designed based on the cDNA sequences of individual firefly species and alignments of firefly species cDNA sequences in the Lampyridae family. Internal primers were based on cDNA sequence alignments of all the species in the Lampyridae family (see Materials and Methods). First strand synthesis and PCR amplification of isolated RNA was attempted 5 times; each time the annealing temperature, primer, and source of RNA were varied. The first PCR reaction (annealing temperature of 52 ºC) using trial 1 RNA with internal, Lampyrinae, and Lucolinae primers gave bands at 600 bp, 1000 bp, and 1500 bp (1500 bp was the expected size for the cDNA) on a 1% agarose gel. This cDNA was stored at –20 ºC for later use. In an attempt to replicate these results, a touchdown PCR (annealing temperature begins at 60 ºC, decreases 1 ºC for each of 12 cycles, and ends with 48 ºC) was done using all primers with trial 1 and trial 4 RNA, but both resulted in smears (representing non-specific annealing of primers producing DNA of different sizes). The third PCR reaction (annealing temperature of 60 ºC) was done with trial 5 RNA and the

internal primers, resulting in no product. This RNA was treated with DNAse and used

along with Trial 1 RNA in the fourth PCR reaction (annealing temperature of 52 ºC) but

bands of the expected size were not observed for either of them. The RNA from trial 1

had most likely degraded at –20 ºC after a few days so the next PCR reaction (Table 2)

was done immediately after the trial 6 RNA extraction. This was a successful PCR

reaction and resulted in products with both the *L. mingrelica* and internal primers.

Additionally, the cDNA that was saved from the first successful PCR reaction (trial 1

RNA with an annealing temperature of 52 ºC) was run on a gel and the band ~1500 bp

was extracted and amplified successfully using the internal primers (see Materials and

Methods).

Table 2: *Primers used for last PCR reaction (successful reactions in bold)*

Primer	Annealing Temperatures
P. myiako and P. rufa alignment	50 ºC, 51.7 ºC
L. noctiluca	50 ºC, 51.7 ºC
L. mingrelica, H. parvula, and H. unmunsana alignment	50 ºC, 51.7 ºC
L. cruciata and L. lateralis alignment	50 ºC, 51.7 ºC
L. noctiluca	50 ºC, 52.8 ºC
L. cruciata	50 ºC, 52.8 ºC
L. mingrelica	**50 ºC, 52.8 ºC**
Internal primers	51.7 ºC, 54.3 ºC, 57.4 ºC, 60 ºC
Internal primers (Gel extracted: Trial 1 RNA)	**51.7 ºC**, 54.3 ºC

The PCR products were of the expected size of approximately 1.65 Kb (Figure 3).

The subsequent steps were carried out using the DNA obtained from this PCR reaction

with primers designed based on *L. mingrelica* luciferase cDNA.
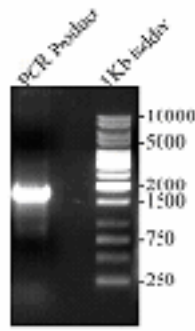
Figure 3. *PCR-amplified cDNA of* L. italica *luciferase on a 1% agarose gel.* Lane 1: PCR product, ~1650 bp in length; Lane 2: 1kb ladder (Fermentas GeneRuler™). PCR was performed as discussed in Materials and Methods.

A portion of the cDNA from successful PCR reactions (Table 2) was transported back to the US on dry ice. The remainder was stored at –20 ºC in Professor Aldo Roda's lab in Bologna. Any unused firefly tails were kept at –80 ºC.

Insertion of L. italica *luciferase cDNA into a pGEX plasmid.* To obtain sequence data for the *L. italica* luciferase cDNA, it was necessary to ligate the cDNA into a pGEX-6P-2 plasmid. We designed *L. mingrelica* primers (used to amplify the *L. italica* cDNA) that were modified on the 5' and 3' ends with extensions containing BamHI and XhoI restriction sites. We initially chose these sites based on what was not present within the *L. mingrelica* luciferase cDNA sequence. Restriction analysis, however, showed that the *L. italica* cDNA contained one of these sites. A digest was carried out using BamHI, EcoRI, SmaI, XhoI, and NotI (each separately) and analysis by electrophoresis revealed that the *L. italica* cDNA contains BamHI and EcoRI sites. We introduced SmaI and XhoI sites into the gene and the PCR product was ligated into the pGEX-6P-2 plasmid (see Materials and Methods).

Sequencing and analysis of cDNA encoding L. italica *luciferase.* The pGEX-6P-2 plasmid containing *L. italica* luciferase cDNA was transformed into *E. coli* cells, plated, and positive bioluminescence was observed. DNA sequencing revealed that the gene was

out of reading frame. Evidently, it was difficult to pick out individual colonies and this colony that was picked was most likely dark. Nonetheless, the full sequence for the *L. italica* luciferase cDNA was obtained (Figure 4). The nucleotide sequence was determined to be 1647 base pairs in length coding for an amino acid sequence of 548 residues. The amplified cDNA product that was sequenced at Bologna University revealed an identical nucleotide sequence.

Figure 4. The nucleotide and deduced amino acid sequences of the *Luciola italica* luciferase cDNA with the start and stop codons boxed. The GenBank® accession number is DQ138966.

Phylogenetic analysis of *L. italica* luciferase showed high percent amino acid identity to the luciferases of other fireflies in the Luciolinae subfamily, bearing the most identity to *H. unmunsana* (95.8%), *H. parvula* (95.6%), and *L. mingrelica* (95.3%). As would be expected, the species that were least similar to the *L. italica* firefly were the click beetles of the Elateridae family and the railroad worms of the Phengodidae family (Figure 5).
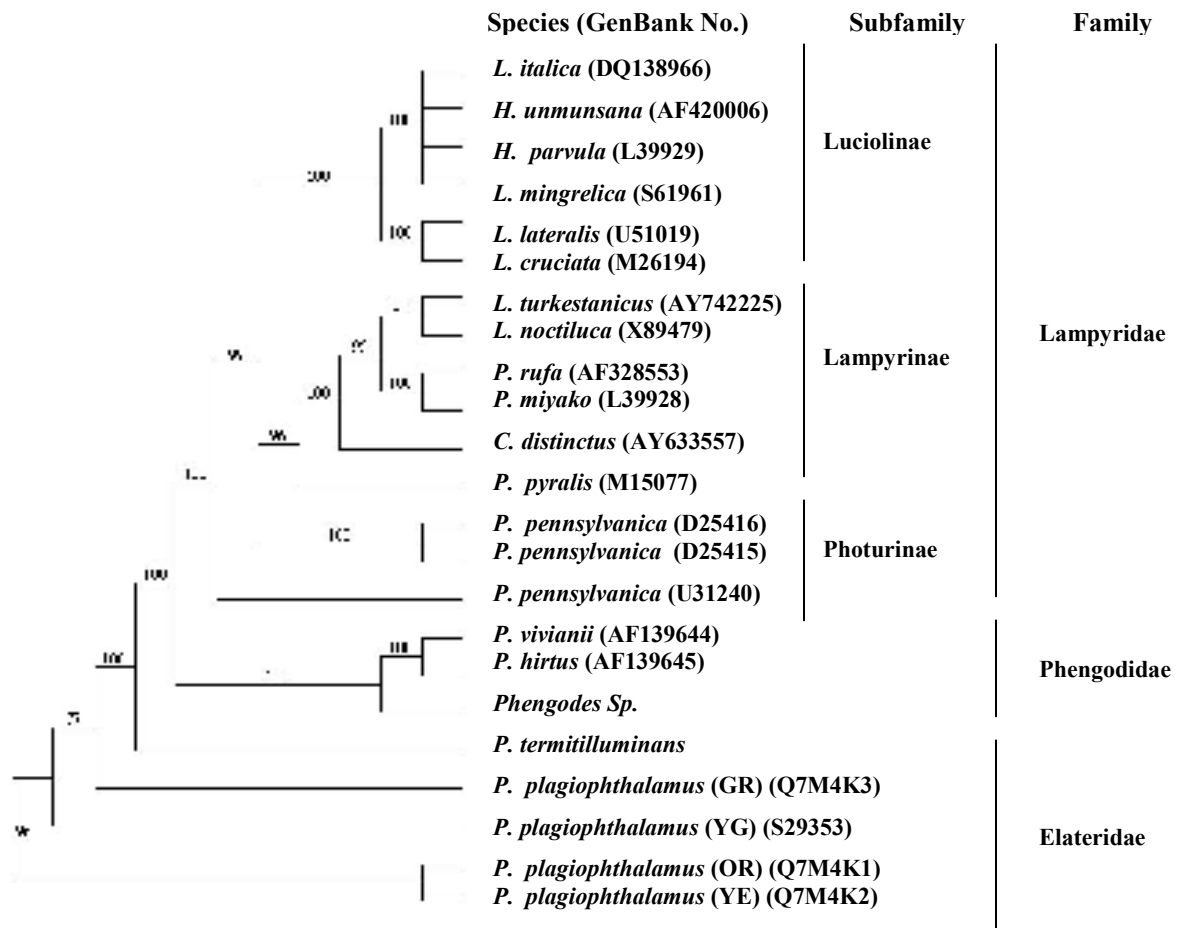
Figure 5. A phylogenic tree (provided by Tara Southworth) of *L. italica* and other known beetle luciferases according to their aligned amino acid sequences. The sequence of *Phengodes* sp. was obtained from Keith V. Wood, personal communication and the sequence of *Pyrearinus termitilluminans* was determined by Viviani *et al.* [20]. All other sequences were obtained from GenBank® with the accession numbers listed in the Materials and Methods. The tree was obtained by bootstrap analysis with the option of heuristic search and the numbers on the branches represent bootstrap values for 2000 replicates. The phylogenic tree was rooted and the yellow and orange isozymes of *P. plagiophthalamus* were selected as the out group based on sequence homology to *L. italica*.

The great similarity between the *L. mingrelica* and *L. italica* fireflies explains why primers based on this species worked for the cDNA amplification. It is interesting to note that the *L. italica* firefly is most similar to the East Asian fireflies *H. unmunsana* (Korea) and *H. parvula* (Japan) and less similar to the Eastern European firefly *L. mingrelica* (Russia) and Western European firefly *L. noctiluca* (Britain). This finding leads us to believe that *L. italica* was an introduced species from East Asia or Eastern Europe.

Analysis of alignments of firefly species (Figure 6) within the Luciolinae subfamily shows that most amino acid differences occur within the N-terminal domain (residues 4-430) but C-terminal regions (residues 440-544) are very similar. Any differences in amino acids that occur in the C-terminal region are generally conservative (the amino acids are similar enough, so they won't change the function of the protein). Unlike *P. pyralis*, the sequences of the species within the Luciolinae family terminate with the amino acids ala-lys-met (AKM). This small sequence functions as a target signal for perioxisomes in mammals so it is assumed that they do the same in fireflies.

```
  1   M E - T E R E E N V V Y G P L P F Y P I E E G S A G I Q L H K Y M Q Q Y A K L -   Lit
  1   . . - M . K . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . -   Hun
  1   . . - M . K . . . . . . . . . . . . . . . . . . . . . . . . . . . . H . . . . -   Hpa
  1   . . - M . K . . . . . . . . . . . . . . . . . . . . . H . . . . . . . . . . . -   Lmi
  1   . . N M . N D . . I . V . . K . . . . . . . . . . . . T . . R . . . E R . . . -   Lcr
  1   . . N M . N D . . I . . E . . . . . . . . . . . . . A . . R . . . D R . . . . -   Lla
  1   . . D A K - - - . I K K . . A . . . . L . D . T . . E . . . . A . K R . . L V P   Ppy

 39   G A I A F S N A L T G V D I S Y Q Q Y F D I T C R L A E A M K N Y G M K P E G H   Lit
 39   . . . . . . . . . . . . . . . . . E . . . . . . . . . . . . . . . . . . Q . . T   Hun
 39   . . . . . . . . . . . . . . . . . E . . . . . . . . . . . . . . . . . . Q . . T   Hpa
 39   . . . . . . . . . . . . . . . . . E . . . . . . . . . . . . . . F . . . . . E .   Lmi
 40   . . . . . . . T . V . . . . . Y . . A E . L E K S . C . G K . L Q . . L V V D . R   Lcr
 40   . . . . . . . T . . . . . Y T . A E . L E K S . C . G . . L . . . L V V D . R   Lla
 38   . T . . . T D . H I E . N . T . A E . . E M S V . . . . . . . R . . L N T N H R   Ppy

 79   I A L C S E N C E E F F I P V L A G L Y I G V T V A P T N E I Y T L R E L N H S   Lit
 79   . . . . . . . . . . . . . M . . . . . . . . . A . . . . . . . . . . . . . . . .   Hun
 79   . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . .   Hpa
 79   . . . . . . . . . . . . . . . . . . . . . . . A . . . . . . . . . . . . . . . .   Lmi
 80   . . . . . . . . . . . . . . I . . F . . G . . . . . . . . . . . . . . . . V . .   Lcr
 80   . . . . . . . . . . . . . . F . . . G . . . . . A . D . . N E . . . . V . .   Lla
 78   . V V . . . . S L Q . . M . . G A . F . . . . . A . D . . N E . . . . L N .   Ppy

119   L G I A Q P T I V F S S R K G L P K V L E V Q K T V T C I K T I V I L D S K V N   Lit
119   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hun
119   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hpa
119   . . . . . . . . . . . . . . . . . . . . . . . . . . K . . . . . . . . . . . . .   Lmi
120   . . . . . S K . . . . . . . . K . . . D . . I T . . . . . T . . . . . . . . . D   Lcr
120   . . . . . S K . . . . . . . . K . . . D . . I T . . . . . A . . . . . . . . . D   Lla
118   M N . S . . . . V . . V . K . . Q . I . N . . . K L P I . Q K . I . M . . . T D   Ppy

159   F G G Y D C V E T F I K K H V E L G F P A T S F V P I D V K D R K H H I A L L M   Lit
159   . . . . . . . . . M . . . . . . . . . . R P . . . . . . N . . Q . V . . . .   Hun
159   . . . H . . M . . . . . . . . . . . . P . . . . . . L . . N . . Q . V . . .   Hpa
159   . . . H . . M . . . . . . . . . . Q P S . . . . . . N . . Q . V . . .   Lmi
160   Y R . . Q . L D . . . . R N T P P . Q . S . . K T V E . - - . . E Q V . . . I .   Lcr
160   Y R . . Q S M D N . . . N T P Q . K G S . . K T V E . - N . . E Q V . . . I .   Lla
158   Y Q . F Q S M Y . . V T S . L P P . . N E Y D . . . - E S F . . D K T . . . I .   Ppy

199   N S S G S T G L P K G V E I T H E G T V T R F S H A K D P I Y G N Q V S P G T A   Lit
199   . . . . . . . . . . . L . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hun
199   . . . . . . . . . . . R . . . . . A . . . . . . . . . . . . . . . . . . . . . .   Hpa
199   . . . . . . . . . . . R . . . . . A . . . . . . . . . . . . . . . . . . . . . .   Lmi
199   . . . . . . . . . . . Q L . . N . . . . . . . . . . R . . . . . . . . . . . . .   Lcr
199   . . . . . . . . . . . Q L . . N A . . . . . . . . . R . . . . . . . . . . . . .   Lla
197   . . . . . . . . . . A L P . R T A C V . . . . . R . . . F . . . I I . D . .   Ppy

239   I L T V V P F H H G F G M F T T L G Y F A C G Y R I V M L T K F D E E L F L R T   Lit
239   . . . . . . . . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . .   Hun
239   . . . . . . . . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . .   Hpa
239   . . . . . . . . . . . . . . . . . . . . . . . . . . V . . . . . . . . . . . . .   Lmi
239   V . . . . . . . . . . . . . . . L I . . F . V . . . . . . . . . . . T . . K .   Lcr
239   . . . . . . . . . . . . . . . . L T . . F . . . . . . . . . . . . T . . K .   Lla
237   . . S . . . . . . . . . . . . . L I . . F . V . L M Y R . E . . . . . . S   Ppy

279   L Q D Y K C T S V I L V P T L F A I L N R S E L L D K F D L S N L T E I A S G G   Lit
279   M . . . . . . . . . . . . . . . . . . . K . . I . . . . . . . . . . . . . . . .   Hun
279   . . . . . . . . . . . . . . . . . . . . K . . I . . . . . . . . . . . . . . . .   Hpa
279   . . . . . . . . . . . . . . . . . . . . K . . I . . . . Y . . . . . . . . . . .   Lmi
279   . . . . . . . . . . . . . . . . . . . . K . . . N . Y . . . . . . V . . . . . .   Lcr
279   . . . . . . S . . . . . . . . . . . . . . . . . . . Y . . . . . . V . . . . . .   Lla
277   . . . . I Q . A L . . . . . . S F F A K . T . I . . Y . . . . H . . .   Ppy

319   A P L A K E I G E A V A R R F N L P G V R Q G Y G L T E T T S A F I I T P E G D   Lit
319   . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hun
319   . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hpa
319   . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . I . . . .   Lmi
319   . . . S . . V . . . . . . . . . . . . . . . . . . . . . . . . . . . . I . . . .   Lcr
319   . . . S . . . . . . . . . . . . . K . . H . . . I . . . . . . . . . . . . . . .   Lla
317   . . . S . . V . . . . . . . K . . H . . . I . . . . . I L . . .   Ppy

359   D K P G A S G K V V P L F K V K I I D L D T K K T L G V N R R G E I C V K G P S   Lit
359   . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . .   Hun
359   . . . . . . . . . . . . . . . V . . . . . . . . . . . . . . . . . . . . . . . .   Hpa
359   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Lmi
359   . . . . . . . . . A . V . . . . . . . . . . . S . . P . . . . V . . . . . . M   Lcr
359   . . . . . . . . . A . V . . . . . . . . . . . P . . . . V . . . . M   Lla
357   . . . . . V . . . . . F . E A . V V . . . . G . . . . . Q . . . L . . R . . M   Ppy

399   L M L G Y T N N P E A T R E T I D E E G W L H T G D I G Y Y D E D E H F F I V D   Lit
399   . . . . . . L . . . . . K . . . D . . . . . . . . . . . . . . . . . . . . . . .   Hun
399   . . . . . . S . . . . . K . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hpa
399   . . . . . . S . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Lmi
399   . . K . . V . . . . . K . L . . . . . . . . . . . . . . . . . E K . . . . .   Lcr
399   . . K . . V D . . . . . I . . . . . . . . . . . . . . . . . E K . . . . . .   Lla
397   I . S . . V . . . . . . N A L . . K D . . . . S . . . A . W . . . .   Ppy

439   R L K S L I K Y K G Y Q V P P A E L E S V L L Q H P N I F D A G V A G V P D S E   Lit
439   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hun
439   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . P Q   Hpa
439   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . P D   Lmi
439   . . . . . . . . . . . . . . . . . . . . . . . S . . . . . . . . . . P V   Lcr
439   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . P I   Lla
437   . . . . . . . . . . . A . . . . . I . . . . . . . . . . . . . L . . D D   Ppy

479   A G E L P G A V V V M E K G K T M T E K E I V D Y V N S Q V V N H K R L R G G V   Lit
479   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hun
479   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Hpa
479   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   Lmi
479   . . . . . . . . . L . S . . N . . . . V M . . A . . . S . A . . . . . . . .   Lcr
479   . . . . . . . . . L . . S . . . . V M . . A . . . S . A . . . . . . . .   Lla
477   . . . . . . A . . . . L . H . . . . . . A . . T T A . K . . . . . .   Ppy

519   R F V D E V P K G L T G K I D A K V I R E I L K K P Q - - - - A K M   Lit
519   . . . . . . . . . . . . . . . . . . . . . . . . . . . - - - - . . .   Hun
519   . . . . . . . . . . . . . . . . . . . . . . . . . . . - - - - .   Hpa
519   . . . . . . . . . . . . . . . . . . . . . . . . . . . - - - - .   Lmi
519   . . . . . . . . . . . . . . . . G R A . . . . . . . V - - - - .   Lcr
519   . . . . . . . . . . . . . . . . G . A . . . . . . . V - - - - .   Lla
517   V . . . . . . . . . . . L . . R K . . . . . I . A K K G G K S . L   Ppy
```

Figure 6. Alignment of the luciferase amino acid sequences (provided by Tara Southworth) of *L. italica* (Lit) *H. unmunsana* (Hun), *H. parvula* (Hpa), *L. mingrelica* (Lmi), *L. cruciata* (Lcr), *L. lateralis* (Lla), and *P. pyralis* (Ppy). The amino acid sequence (single letter codes) of *L. italica* luciferase is shown and the dots represent identical amino acid residues.

26

Realignment and verification of Luciola italica *luciferase cDNA sequence.* To

express the protein in *E. coli*, the *L. italica* luciferase cDNA needed to be re-inserted into

the pGEX-6P-2 plasmid so it would be in the correct reading frame. Additionally, we

wanted the *L. italica* cDNA between BamHI and XhoI restriction enzyme sites so it

would be in the same region of the pGEX-6P-2 plasmid as *P. pyralis* is. The internal

BamHI site already present in the cDNA was removed by a silent mutation and a BamHI

site was introduced into the plasmid immediately preceding the ATG start codon (see

Materials and Methods). Upon digestion with BamHI and XhoI, the excess unwanted

region before the *L. italica* cDNA was cut away and it was reinserted back into the

pGEX-6P-2 plasmid (Figure 7).
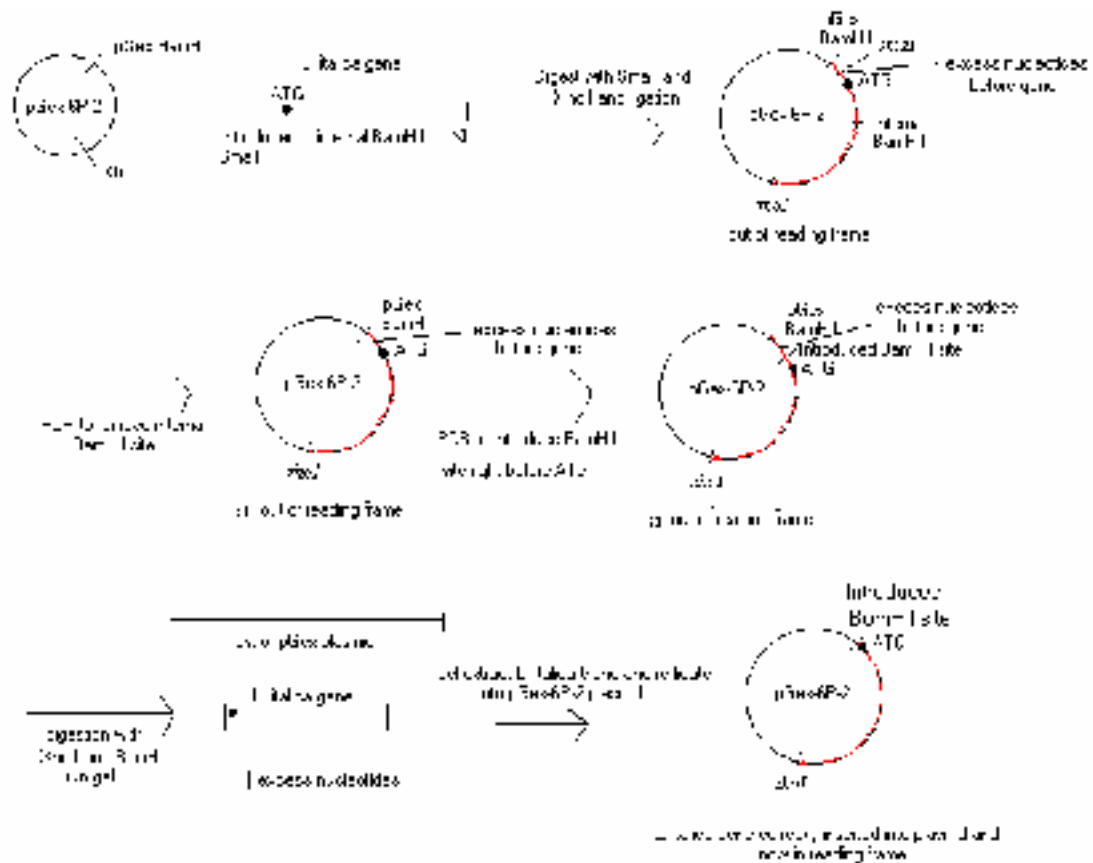
Figure 7. An illustration for the re-alignment of the *L. italica* cDNA in the pGex plasmid (plasmid diagrams are not drawn to scale).

The gene was now in reading frame so positive bioluminescence was observed

upon treatment of XL-10 Gold *E. coli* cells with 1 mM luciferin (Figure 8). The color

expressed in XL-10 *E. coli* cells is not the true color; *L. italica* luciferase actually emits a

more yellow light.
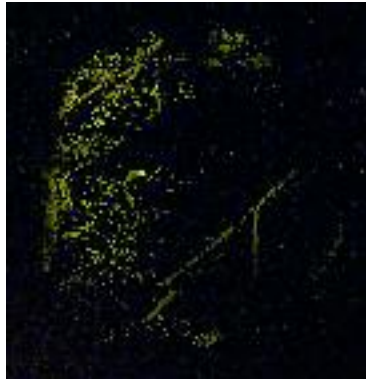
Figure 8. Image of bioluminescence emitted by XL-10 Gold *E. coli* colonies expressing the *L. italica* luciferase gene as a GST-fusion protein. The image was obtained with a 16 s exposure using a Kodak DC290 Digital Camera equipped with accessory a macro-imaging option.

The aligned luciferase cDNA was sequenced, which showed that it was properly

inserted in the pGex plasmid (Figure 9).
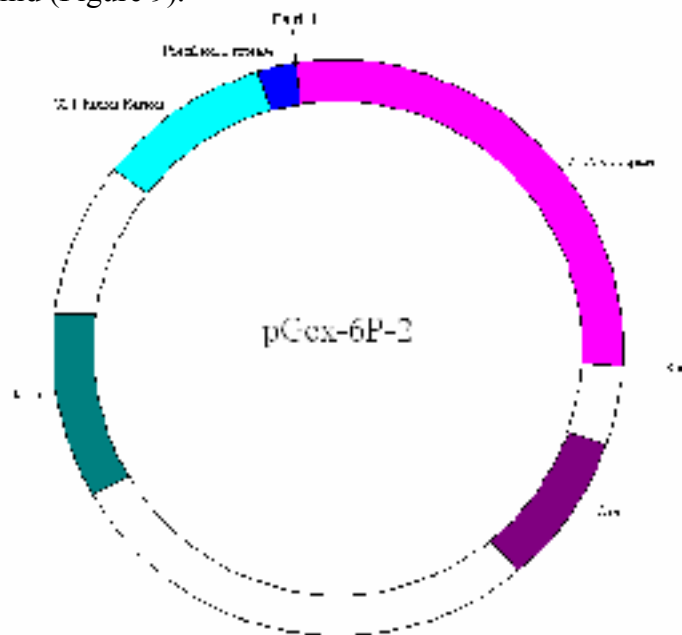
Figure 9. Diagram of the pGEX plasmid containing the *L. italica* lucfierase cDNA, which was inserted into the multiple cloning region between BamHI and XhoI restriction sites.

Determining optimum conditions for expression of Luciola italica *protein.*

Previous isolations of the *L. italica* luciferase protein had resulted in relatively low yields

(< 5 mg for a 500 mL culture). It was hypothesized that this low yield was due to a

significant amount of improperly folded protein that formed inclusion bodies during the

bacterial growth stage. Bacteria grow more slowly at lower temperatures so we

hypothesized that by growing *E. coli* at a lower temperature the *L. italica* luciferase

protein would have more time to fold properly. Cultures of *E. coli* cells (5 mL)

containing the pGEX-6P-2 plasmid expressing the *L. italica* luciferase gene were grown

at 18 ºC for different lengths of time (ranging from 17 hrs-27 hrs) after being induced

with 0.1 mM IPTG. The cultures were partially purified, up to the step of the addition of

20% Triton X-100 and activity measurements were made for the crude lysates of each

time point (see Materials and Methods). These measurements were used to estimate the

amount of the luciferase-fusion protein present in the 5 mL culture (i.e. the higher the

estimated activity, the greater the amount of fusion-protein present). It can be seen that

as the length of induction time increased the estimated activity increased and began to

level off after about 25 hrs (Figure 10 A). When a 250 mL culture was grown under

these same conditions, however, the yield was not better than it had been in previous

purifications (~2.4 mg).

Experiments were done using the same procedure with an induction temperature

of 22 ºC and a range of induction times between 4 hrs and 36.5 hrs. The estimated

activity increased as the induction time increased (Figure 10 B). The *L. italica* luciferase-

fusion protein was obtained in the highest yield after growing *E. coli* at 22 ºC for around

30 hrs. Because of this data, we will now grow the *L. italica* luciferase protein at 22ºC between 25-30 hrs.

This experiment was repeated for the *P. pyralis* luciferase protein and results showed that the highest yield is obtained with growth at 22ºC for about 18 hrs after induction with IPTG. Also, it showed a temporary decrease in yield around 22 hrs, followed by an increase, and leveled off. It is interesting to note that despite the close similarity in structure between *L. italica* and *P. pyralis* proteins, *L. italica* takes a significantly longer time to fold.
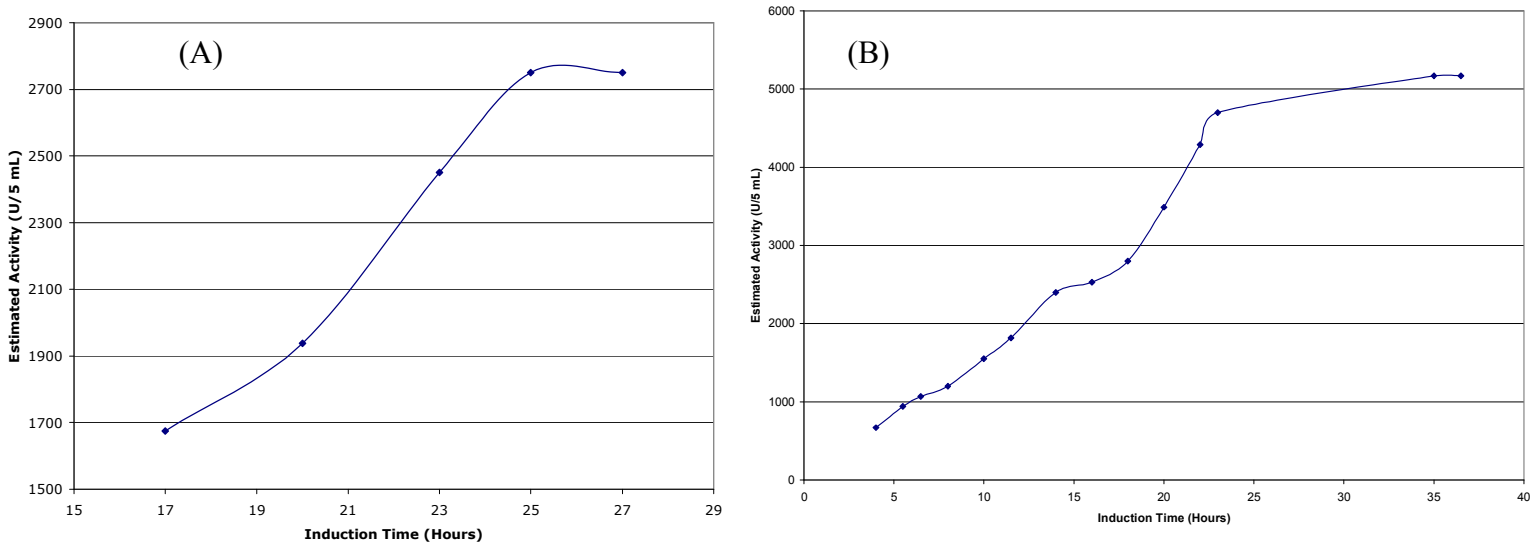
Figure 10. Relative activity measurements for different induction times. (A) **18 ºC**: The *L. italica* luciferase protein was grown in *E. coli* cells at 18 ºC for different periods of time after being induced with 0.1 mM IPTG and was purified on a small scale (5 mL of bacterial culture) up to the point of the addition of triton (see Materials and Methods). Relative activity of the crude lysates were measured and plotted against the length of time grown after induction. (B) **22 ºC**: The same procedure was followed with an induction temperature of 22 ºC.

Characterization of L. italica luciferase. The newly purified *L. italica* luciferase

protein was characterized for its light emission properties, steady-state kinetics, and

specific activity (Table 3).

Table 3. *Properties of* Luciola italica *luciferase compared to* Photinus pyralis *luciferase.*

Protein	Relative Specific Activity (%WT)		$kcat$ (s^{-1})	Km (µM)		Bioluminescence Emission Maxima, pH 7.8			Decay Time (min)
-	Flash Height (units/mg)	Integrated 15 min (RLU/mg)	-		-	Maxima (nm)	Bandwidth		-
-	-	-	-	LH_2	MgATP	-	50%	20%	-
P.pyralis	135,000 (100)	1.78×10^8 (100)	0.167	15	160	557	66	113	0.20
L. italica	104,000 (77)	3.83×10^8 (215)	0.127	104	180	564	89	109	0.11

The specific activity of an enzyme is the amount of substrate converted to product

per unit time (defined as enzyme units) per mg of enzyme. The flash height specific

activity represents the specific activity at the maximum emission of light (when the

enzyme is working its best). The flash height activity of *L. italica* luciferase is ~77% that

of *P. pyralis* luciferase activity, showing that it emits a slightly less intense light.

When looking at the light emission profiles of *L. italica* and *P. pyralis* luciferases

(Figure 11), it is interesting to note the difference in activity decay patterns.
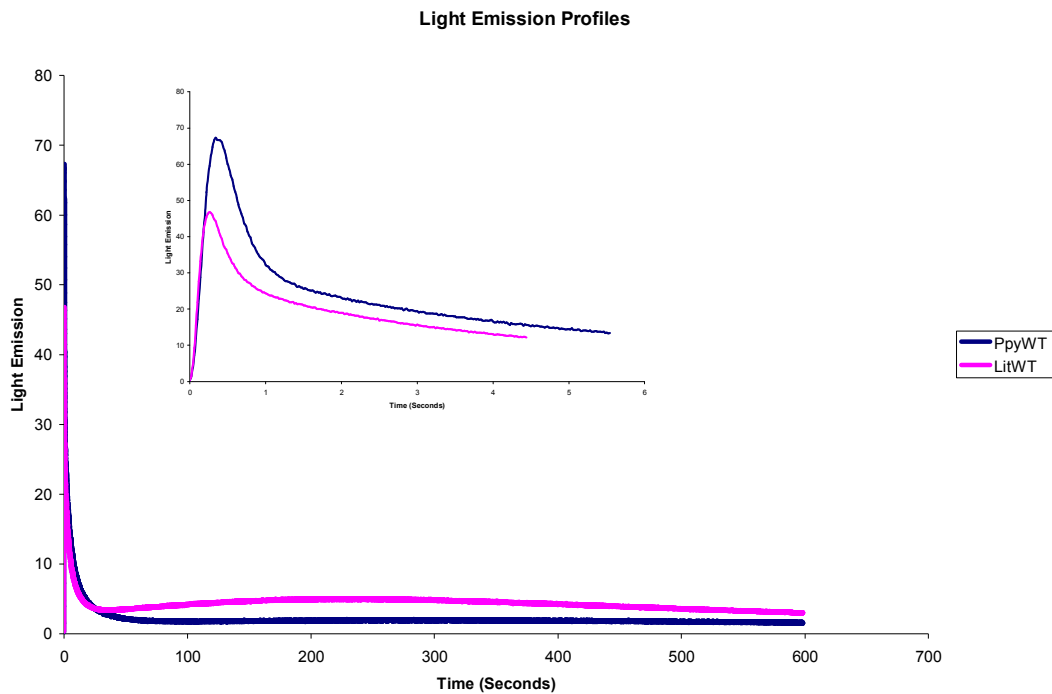
Light Emission Profiles

Figure 11. Light emission profiles of *L. italica* (pink) and *P. pyralis* (blue) luciferases at pH 7.8. The assays were prepared as previously described in materials and methods. An expansion of maximum light emission is shown as well.

While *P. pyralis* luciferase undergoes a steady decline, *L. italica* luciferase experiences a slight increase in activity giving a second, smaller emission of light. Although *L. italica* decays more quickly to 20% of its maximum activity, the light lasts longer at a visible intensity because of this second activity increase. This accounts for the ~215-fold greater integrated specific activity of *L. italica* compared to *P. pyralis*. This would be a benefit in reporter gene assays, where the light is measured after it reaches a maximum emission.

The K_m value of an enzyme reflects the affinity of an enzyme for a particular substrate and how well that enzyme catalyzes the formation of product. The K_m value is the concentration of substrate at half the V_{max} (the greatest rate of catalysis because the enzyme is saturated with substrate). It is determined by measuring enzyme activity, keeping the concentration of one substrate constant while varying the concentration of the other. Enzyme activity is measured until it reaches a constant value, indicating that the

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V_{max} has been reached. This data is plotted and the K_m is obtained from the Michaelis-Menton graph (Figure 12).
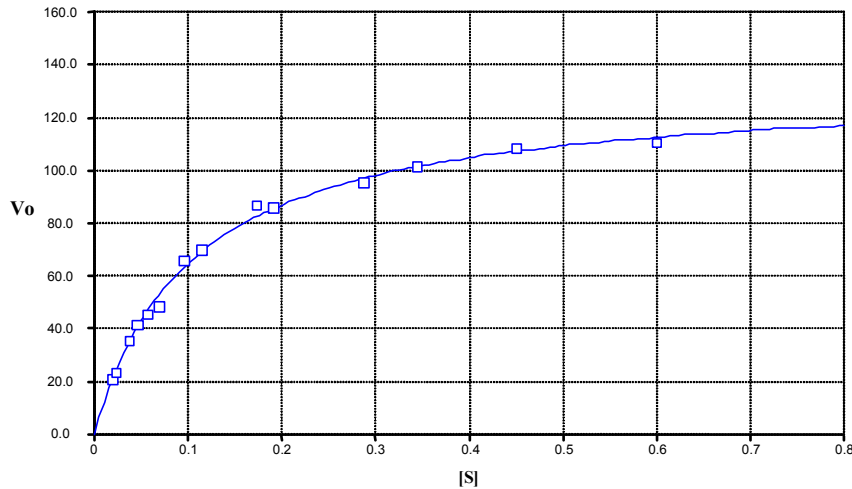

Figure 12. Michaelis-Menton curve for LH_2 for *L. italica* at pH 7.8. Concentration of the substrate ([LH_2]) is in mM. This graph was plotted using Enzyme Kinetics Pro 2.34 (materials and methods).

The *L. italica* protein has almost a 7 fold greater K_m for LH_2 but only a slightly higher K_m for Mg-ATP. Thus, the amount of LH_2 needed to saturate the *L. italica* protein is much greater than that for *P. pyralis* (270 µM vs. 70 µM). This is because *L. italica* has a lower affinity for both substrates and it takes a longer time for the substrate to bind at lower concentrations.

The wavelengths where the maximum emission of light from the *L. italica* luciferase reaction occurs were observed at different pHs (Figure 13).
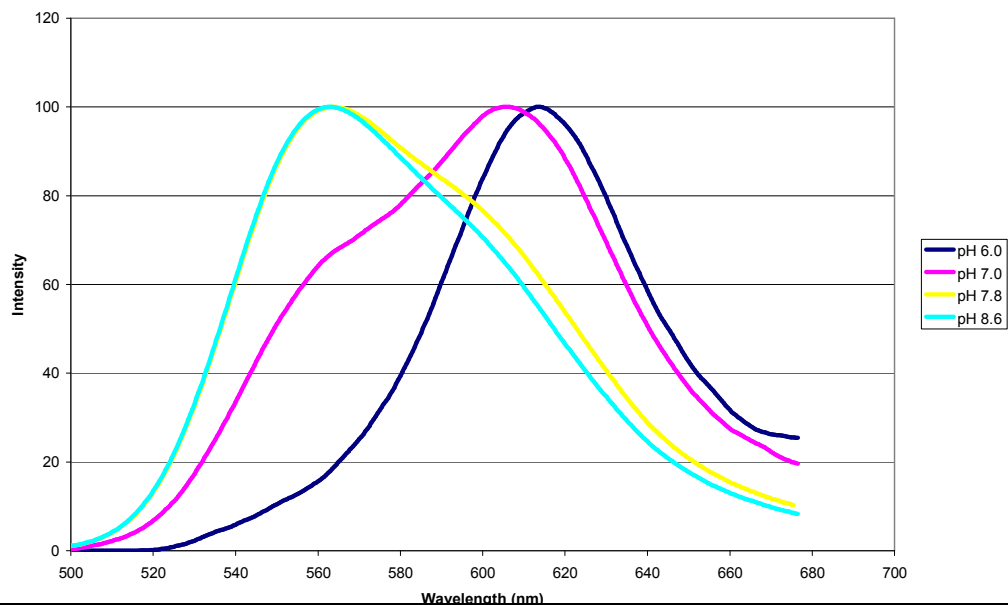
-	pH 6.0			pH 7.0			pH 7.8			pH 8.6		
Enzyme	Maximum Emission	Bandwidth		Maximum Emission	Bandwidth		Maximum Emission	Bandwidth		Maximum Emission	Bandwidth	
-	-	50%	20%	-	50%	20%	-	50%	20%	-	50%	20%
Lit WT	613 nm	59	112	603 nm	96	143	564 nm	89	109	563 nm	82	535
Ppy WT	612 nm	61	100	561 nm	102	148	557 nm	73	119	556 nm	65	108

Figure 13. Bioluminescence spectra for *L. italica* and *P. pyralis* luciferase proteins. Spectra were obtained for both *L. italica* and *P. pyralis* in buffers of different pHs (6.0, 7.0, 7.8, and 8.6). The wavelengths at which maximum emission occurred were observed (see Materials and Methods). Values of emission maxima for *P. pyralis* and *L. italica* are reported in this table. The spectral emissions of *L. italica* at different pHs are also shown here (above).

When comparing the maximum spectral emissions of *L. italica* luciferase to *P. pyralis* luciferase it can be seen that *L. italica* light emissions are slightly red-shifted. At pH 6.0, *L. italica* emission is similar to *P. pyralis* while at pH 7.8, *L. italica* is bimodal and re-emits light around 610 nm (Figure 14).
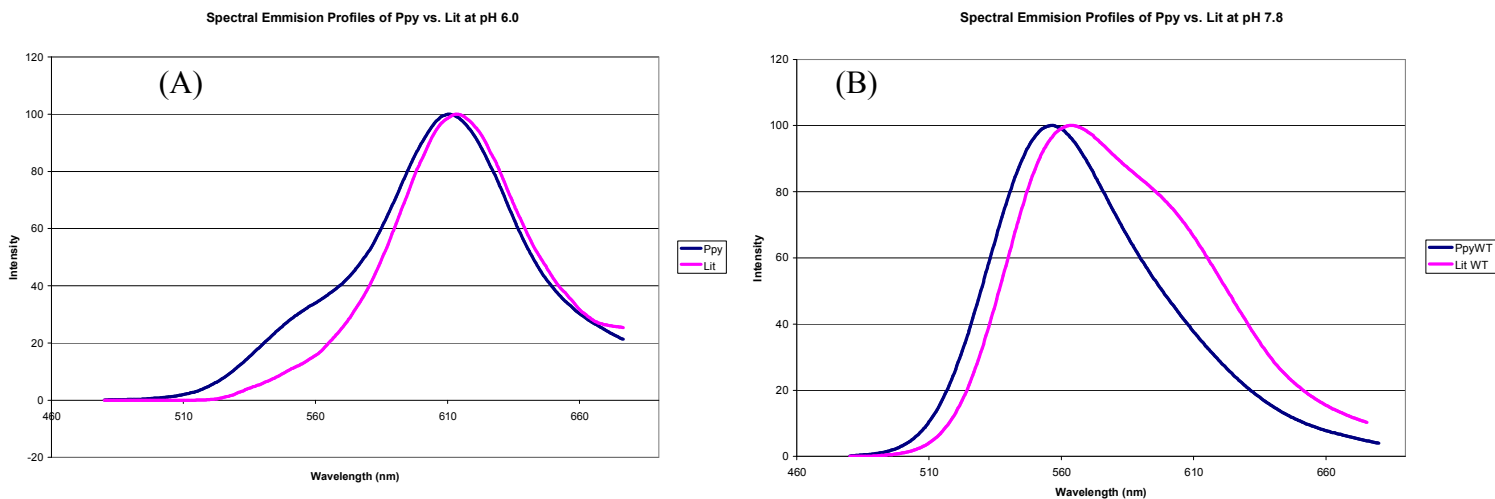
Figure 14. Spectral emission comparisons between *L. italica* and *P. pyralis* at pH 6.0 (A) and 7.8 (B).

While the maximum peaks for *L. italica* protein emissions and *P. pyralis* emissions at pH 7.8 are similar, it is the wider bandwidth of the *L. italica* emission curve that gives it its yellow color (Figure 15).
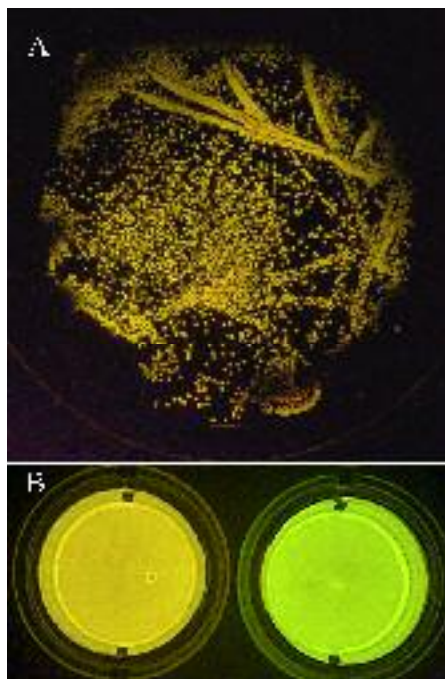

Figure 15. Images of bioluminescence emission (provided by Tara Southworth): (A) colonies of *E. coli* expressing *L. italica* luciferase as a GST-fusion protein and (B) *in vitro* reactions of purified *L. italica* (left) and *P. pyralis* (right). The *in vitro* reactions in 25 mM glycylglycine buffer, pH 7.8 (1 ml) contained 5 μg of purified enzyme in CBA, 270 or 70 μM of LH_2 and 2 mM Mg-ATP. All images were obtained with a 16 s exposure using a Kodak DC290 digital camera equipped with a macro-imaging accessory.

CONCLUSION

The cloning, expression, and characterization of *L. italica* luciferase have been accomplished. We are currently working on further characterizing the *L. italica* luciferase protein with assays such as the CD spectrum assay, fluorescence denaturation assay, and thermo-stability assays. Also, it is necessary to determine the long-term storage conditions for the *L. italica* protein because they denature and come out of solution after only a few months of storage.

The *L. italica* reaction sustains its light for a longer period of time than *P. pyralis*; this would be beneficial in reporter gene assays. We are working on mutating the *L. italica* protein to make it more thermostable and emit a bright red light. This has been done successfully with *P. pyralis* [9, 10] so we are introducing the same mutations into *L. italica*. Lastly, the *L. italica* gene has been prepared for insertion into a Promega mammalian expression plasmid by removal of the terminal perioxisome site (ala-lys-met) in the gene (see Materials and Methods). With its prolonged light activity, this enzyme seems to be a promising find.

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