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Studies on bioluminescence in different marine bacteria with focus on luciferase and the impact of LuxF on light intensity

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Abstract

Bioluminescence is defined as the enzymatic production of light in living organisms. Different organisms ranging from microbes to animals have this ability, though abundance is especially high in the marine environment. This master thesis focused on the bioluminescence in marine bacteria which can be found free-living or in symbiosis with fish and other sea life. The reaction producing light is catalysed by a flavin-dependent monooxygenase, bacterial luciferase. However, other structural and regulatory genes are involved in the process. These structural genes are organised in a single operon *luxCDAB(F)EG.* In this study, special attention is drawn to the heterodimeric bacterial luciferase (encoded by *luxAB*) and the influence of *luxF*, which is only present in some *Photobacterium* species, on total light emission.

Luciferase from four marine bacteria was produced recombinantly in *Escherichia coli* and purified using affinity chromatography. Different approaches were tested in order to find an ideal experimental procedure leading to native heterodimer. All luciferases could be purified in their native form; however, yields were very low. Three of the four enzymes were proven to be active in an *in-vitro* assay. Analytical size exclusion chromatography showed that pure protein was obtained, although a majority was present in aggregates. For this reason, a realistic comparison of the bacterial luciferases could not be achieved.

The *lux* operons of *P. leiognathi* ATCC 25521 and *P. mandapamensis* ATCC 27561 were compared in an *in-vivo* assay. The genes *luxCDAB(F)EG* were expressed recombinantly in *E. coli* and exclusion of *luxF* led to a decrease in light emission. This supported prior studies, which found that LuxF acts as a scavenger of an inhibiting side product (an FMN derivative) produced during the light-emitting reaction.

Zusammenfassung

Biolumineszenz wird definiert als die enzymatische Produktion von Licht durch Lebewesen. Verschiedenste Organismen, von Mikroben über Pilze bis zu Tieren, besitzen diese Fähigkeit, welche vor allem im Meer in großer Vielfalt vorkommen. Der Fokus dieser Masterarbeit liegt auf den biolumineszenten Meeresbakterien, welche sowohl freilebend als auch in Symbiose mit Fischen und anderen Meereslebewesen vorgefunden werden. Die lichtproduzierende Reaktion wird von einer flavinabhängigen Monooxygenase katalysiert, der bakteriellen Luziferase. Jedoch sind auch andere strukturelle und regulatorische Gene in diesen Prozess involviert. Die strukturellen Gene liegen in einem einzigen Operon *luxCDAB(F)EG*. Den Schwerpunkt dieser Arbeit bildete die heterodimere Luziferase (codiert von *luxAB*) und der Einfluss von *luxF* auf die Lichtausbeute. *LuxF* kommt nur in manchen Arten der Gattung *Photobacterium* vor.

Die Luziferase von vier Meeresbakterien wurde rekombinant in *Escherichia coli* produziert und mithilfe der Affinitätschromatographie gereinigt. Verschiedene Methoden wurden getestet, um eine ideale Vorgehensweise zu finden, welche zu einer Anreicherung der heterodimeren Luziferase führten. Dabei konnten alle Luziferasen in nativer Form gereinigt werden, allerdings mit sehr geringer Ausbeute. In einem *in-vitro* Test waren drei dieser vier Luziferasen aktiv. Analytische Größenausschlusschromatographie zeigte, dass zwar reines Protein gewonnen wurde, das meiste davon jedoch aggregiert vorlag. Daher war kein zuverlässiger Vergleich der bakteriellen Luziferasen möglich.

Die *lux* Operons von *P. leiognathi* ATCC 25521 und *P. mandapamensis* ATCC 27561 wurden *in-vivo* verglichen. Die Gene *luxCDAB(F)EG* wurden rekombinant in *E. coli* exprimiert, dabei führte der Ausschluss von *luxF* zu einer signifikant geringeren Lichtemission im Beobachtungszeitraum. Das bekräftigte frühere Studien, welche zeigten, dass LuxF ein inhibierendes Nebenprodukt der Lichtreaktion (ein FMN-Derivat) binden kann.

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1 Introduction

1.1 Bioluminescence

Bioluminescence is the production of light by living organisms in an enzyme-dependant reaction. The abundance of luminescent species is extraordinarily high, especially in the ocean. They do not occur exclusively in the deep sea, where no sun light arrives, but are spread throughout oceans from surface to sea floor, through warmer and cooler waters. In fact, this causes bioluminescence to be the primary source of light in the majority of the ocean. In the marine environment, luminous organisms dominate regarding biomass, and bacteria and dinoflagellates dominate in abundance (1, 2). Three basic biological functions associated with bioluminescence have been observed: food procurement by either attracting prey or locating it, communication, including mating, and defence against predators (3). These functions apply mostly to higher organisms such as squid, fish and crustaceans, and most of these are able to produce light themselves (1). Anyway, some fish and squid species profit from bacterial symbionts cultivated in specialized light organs. The benefits for the involved microbes, like *Photobacterium leiognathi*, *P. mandapamensis* or *Aliivibrio fischeri*, in this symbiosis are still uncertain (4–6). It is believed that the host provides an ideal growth environment for the bacteria (7). This is especially important, as bacterial bioluminescence in some cases is triggered only after an adequate cell density is reached. Luciferase levels are increased 100- to 1000-fold compared to lower population densities, resulting in an up to 10^6-fold increase in light emission. The resulting glow is continuous as long as oxygen is available. Secondary metabolites act as signal molecules and are responsible for this effect, originally referred to as autoinduction. These metabolites (autoinducers) activate or derepress the *lux* operon transcription, regulating the gene expression. These regulation systems are now referred to as quorum sensing, which reflects the ability of bacteria to detect population density (7–9).

Not all luminous bacteria found in the ocean live in the light organs of higher organisms. The phenomenon called “milky sea”, which describes a luminescent glow on the surface of the ocean, has been observed for centuries. In 2005, the first satellite picture of this event was reported, where an area of 15400 km^2 was glowing continuously for three nights (10). It is suspected that the luminescence is caused by bacteria (*Vibrio harveyi*) colonizing the microalga *Phaeocystis* (11). Another example of free-living luminous bacteria are strains found in the guts and on excreted faecal matter of fish (12). The benefit these free-living microbes might gain from the utilization of bioluminescence is not as apparent as for bacteria living in light organs. The most popular hypothesis suggests that the glow is used to attract fish, which then ingest the potential food particles the bacteria grow on. In the intestines of the fish, the bacteria

find a beneficial environment and are then distributed by excretion, allowing intensive proliferation (1, 13).

It is apparent that not all luminous bacteria exist in the same conditions regarding for example water temperature and living environment (free or in light organs). However, all of the bacterial strains exhibiting bioluminescence (Figure 1) were shown to be gram-negative, motile rods and facultatively anaerobic (13–15).

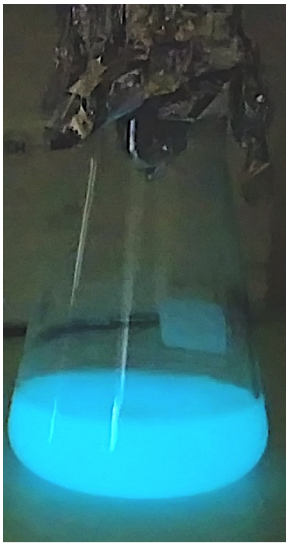

Figure 1: *Photobacterium mandapamensis* culture in sea water medium.

1.2 Bioluminescence Reaction

The bioluminescence reaction (Scheme 1) in bacteria is catalysed by the bacterial luciferase, a heterodimeric monooxygenase. The reaction involves the oxygenation of an aliphatic aldehyde to the corresponding fatty acid under use of molecular oxygen and reduced flavin mononucleotide ($FMNH_2$) (7). During the reaction, an excited state flavin derivative FMN-4a-hydroxide is formed (Figure 2). This molecule acts as the luciferin in bacterial bioluminescence by releasing free energy when it relaxes to its ground state (16). This energy is released as light with a maximum wavelength of 490 nm.

$$FMNH_2 + RCHO + O_2 \xrightarrow{LuxAB} FMN + RCOOH + H_2O + h\nu\ (\sim 490\ nm)$$

Scheme 1: General scheme of the reaction catalysed by bacterial luciferase (LuxAB).

The natural aldehyde substrate for this reaction is tetradecanal, though bacterial luciferase accepts a variety of long-chain aldehydes, even unsaturated aldehydes (17–19).

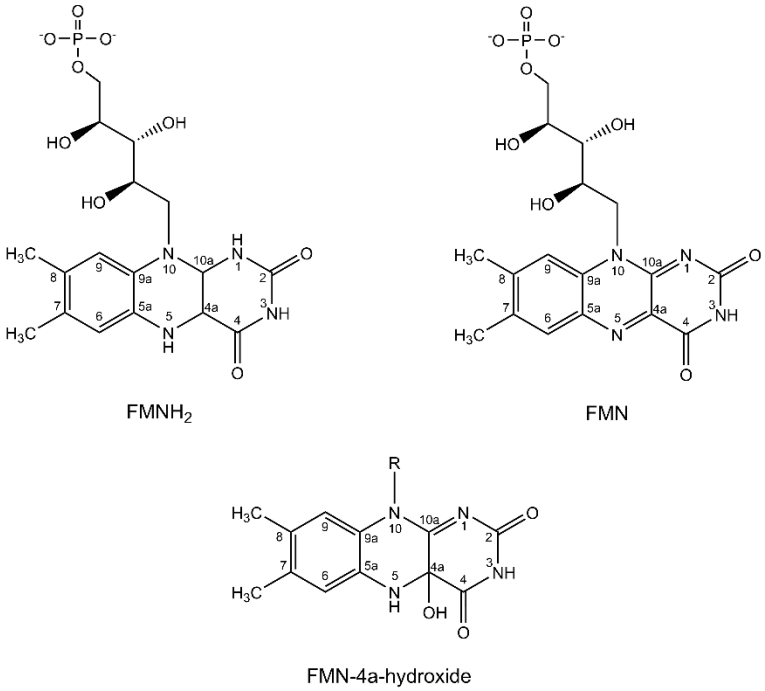

Figure 2: Structure of important flavin derivatives involved in the bioluminescence reaction. Reduced FMN ($FMNH_2$) is the substrate and is oxidized to FMN during the reaction. FMN-4a-hydroxide is considered as the actual luciferin by emitting light when relaxing from its excited state to the ground state.

1.3 Organisation of the *lux* Operon

The genes required for bacterial bioluminescence have already been described in 1985. Five genes from *A. fischeri* were identified to be responsible for light production: *luxC, luxD, luxA, luxB* and *luxE* (20). It is now established that the *lux* operon in all marine bacteria is comprised of six core genes *luxCDABEG* (7, 21). The genes *luxA* and *luxB* encode the two subunits α and β of bacterial luciferase, *luxC*, *luxD* and *luxE* make up the fatty acid reductase complex responsible for the generation of aldehyde substrate and *luxG* encodes a flavin reductase. In addition, some *Photobacteria* species also carry the gene *luxF* between *luxB* and *luxE* (22). A general depiction of the core genes constituting the *lux* operon is shown in Figure 3.

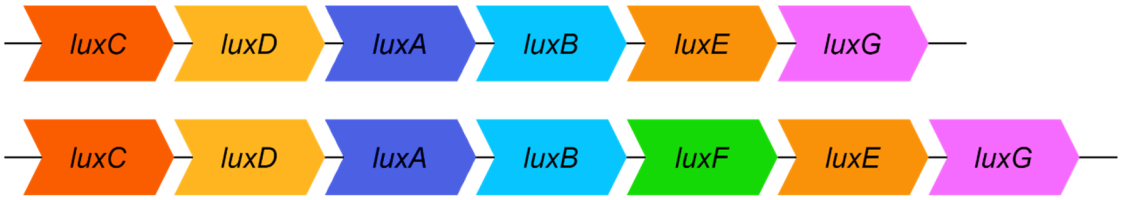

Figure 3: Structural genes of the *lux* operon in luminous marine bacteria. *LuxA* and *luxB* encode the two subunits of bacterial luciferase, *luxC*, *luxD* and *luxE* encode the fatty acid reductase complex, *luxG* encodes a flavin reductase. Some luminous bacteria have an additional gene *luxF*.

1.3.1 Regulatory Genes

Apart from these core genes, several genes linked to the *lux* operon have been found upstream and downstream of the structural genes. They were associated with the complex regulatory mechanisms involved in bacterial bioluminescence and studied especially in *V. harveyi* and *A. fischeri* (20, 23). Regulation of bioluminescence has already been mentioned above, regarding the cell density. This relationship can be explained with the accumulation of the autoinducer, which initiates light production upon reaching a certain threshold concentration in the media. In *A. fischeri*, two genes compose one of the quorum sensing systems regulating bioluminescence. *LuxI* was found within the *lux* operon located before *luxC* and encodes for the autoinducer synthase. The gene *luxR* is located directly upstream of the *lux* operon with an inverse reading frame and encodes a transcription factor. LuxR is autoinducer-dependent and in complex with the product generated by LuxI induces the transcription of the *lux* operon (20, 24). The LuxR-LuxI system is only one of many regulatory mechanisms. For example, it was reported that bioluminescence is repressed under reducing conditions by the ArcAB system in *A. fischeri* (25).

A similar regulatory mechanism was reported in *V. harveyi,* which lacks the gene for LuxI in the location upstream of *luxC*. Regulatory regions in *V. harveyi* have been found at a different locus than the *lux* operon (26). This region was later identified as a single *luxR* gene which is not homologous to that of *A. fischeri*, but also encodes a transcriptional activator (27, 28). In this regulatory mechanism, complex phosphorylation/dephosphorylation cascades and small regulatory RNAs (sRNAs) are involved in the control of light production (29).

1.3.2 Other Associated Genes

Photobacterium species have been found to harbour additional genes downstream of the *lux* operon. The four genes *ribEBHA* immediately adjacent to *luxG* have been identified to be involved in the riboflavin biosynthesis. Between the *lux* and *rib* genes, no transcriptional terminator region was found. It was hypothesized that the *rib* genes could be part of the same transcriptional unit and would therefore be co-expressed. As riboflavin is a precursor of FMN, which is essential for the bioluminescence reaction, the *rib* genes might facilitate light production (30–32). Similarly, *ribB* (formerly named *luxH*) was reported to be part of the *lux* operon in *V. harveyi*, also found directly downstream of *luxG*. However, for this strain, a termination signal was found directly after *ribB* (33).

1.4 LuxAB

The bacterial luciferase (LuxAB) is a heterodimeric monooxygenase with a molecular weight of around 80 kDa, depending on the species. The two subunits α (40-44 kDa) and β (35-40 kDa) fold into a single domain $(\beta/\alpha)_8$ or TIM barrel and share a sequence similarity of about 30 % (34, 35). Cline and Hastings have already suggested in 1972, that the active side resides on the α-subunit and the β-subunit mainly provides stability, particularly stabilizing the active conformation of the α-subunit (36). It was found that both subunits are active even in the absence of the other monomer. Anyway, the specific activity for the individual subunits is much lower compared to the natural heterodimer (37, 38).

Interestingly, subunits which were synthesized and allowed to fold separately, did not assemble into the natural active heterodimer. Instead, they had to be denatured prior to renaturation in order to yield correctly assembled luciferase (39). To elucidate if one or both subunits are responsible for this inability to assemble correctly, refolding experiments with combinations of native and unfolded α- and β-subunits were conducted. The results indicate that native β-subunit prevents the correct quaternary structure by forming a homodimer in the absence of α. This stable homodimer β_2 is then unable to associate with α to form the heterodimer αβ. Instead, it folds into a kinetically trapped alternative to the active conformation via kinetic partitioning during folding. However, the native quaternary structure αβ of bacterial luciferase is kinetically preferred over the homodimer and this folding reaction is under kinetic control (40, 41).

The first crystal structure of a bacterial luciferase was solved in 1995 from the marine bacterium *Vibrio harveyi* (34). To date, this is the only species of which the luciferase structure was reported. However, one year later the same protein has been crystallized again and the structure could be refined (42). Another step in determining the native state and the active centre was the crystal structure with bound FMN, which was solved over 10 years after first x-ray structure. The structural evidence found in this study confirms the location of the active site at the α-subunit (43). A loop on the α-subunit connecting β-strand 7 to α-helix 7 (residues 262-290) was disordered to different extend in all crystal structures. This loop comprises a region of about 25 residues labile to protease degradation, leading to activity loss (44, 45). The binding of FMN or phosphate was demonstrated to protect against proteolytic inactivation of the enzyme. This is due to a small conformational change in the loop upon binding of the substrate, which decreases the access of proteases to this labile region (44, 46, 47).

As already discussed above, the individual subunits of bacterial luciferase can produce light, but at a much lower intensity than the native heterodimer (37, 38). As the active site of the

enzyme is found on the α-subunit, but the heterodimer is so much more active, the role of the β-subunit in stabilizing the complex needed to be addressed. First structural evidence was found based on the crystal structure of the luciferase/FMN complex (Figure 4). A residue of the mobile loop (Phe272), not detected in the previous structures, was observed to be in contact with Tyr151 of the β-subunit. This tyrosine residue was mutated to five different amino acids. These substitutions all resulted in decreased affinity for $FMNH_2$, hence activity was lost. Based on these findings it was suggested that the β-subunit stabilizes the active form of the α-subunit (43).

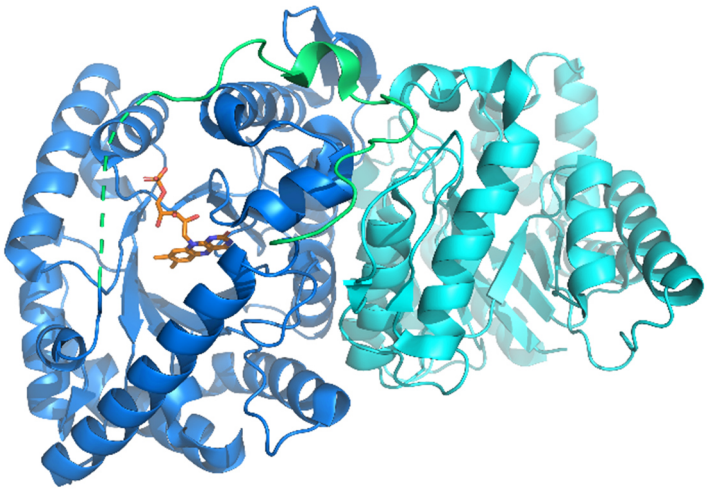

Figure 4: Structure of bacterial luciferase from *Vibrio harveyi* with FMN (PDB: 3FGC). The α-subunit is shown in blue with the mobile loop in green, the β-subunit is depicted cyan. FMN (orange) is located in the active site.

The structure of the β_2-homodimer could also be solved. Due to the high sequence similarity of the subunits, the general arrangement is very similar to the heterodimer. Salt links and hydrogen bonds at the subunit interfaces of the hetero- and homodimer were compared. The homodimer was found to have a smaller interface and therefore has less favourable energy, which is consistent with the theory that the heterodimer is thermodynamically more stable (40, 48).

1.5 Other Structural Proteins

1.5.1 LuxCDE

The generation of the aldehyde substrate is catalysed by the fatty acid reductase complex. Three enzymes, encoded by the genes *luxC*, *luxD* and *luxE,* are involved in this reaction: reductase, transferase and synthetase (49). Biochemical experiments suggested that the three proteins are arranged in a multienzyme complex composed of tetrameric reductase, four polypeptides of synthetase and two to four subunits of transferase (50). To this date, only the crystal structure of the transferase LuxD is available, which was also the first solved structure of a thioesterase. This structure together with site-directed mutagenesis lead to the conclusion that the residue Ser114 has catalytic function and is part of a lipase-like catalytic triad (51). Structures of LuxC and LuxE have not been solved yet, let alone the multienzyme complex as a whole. However, homology models of those enzymes were generated and a $LuxC_4LuxD_4LuxE_4$ complex was constructed, also taking into account prior biochemical characterisations (52).

The synthesis of aldehydes required for the bioluminescence reaction is based on the reduction of fatty acids utilizing ATP and NADPH. The first step is catalysed by acyl-transferase LuxD, which cleaves the acyl group from acyl-ACP (acyl carrier protein) or acyl-CoA (53). The acyl-protein-synthetase (LuxE) then activates the fatty acid with ATP, resulting in an acyl-AMP intermediate bound to the enzyme. The acyl group is then transferred to the acyl-CoA-reductase (LuxC), where it is reduced to the corresponding aldehyde by NADPH (15, 54).

1.5.2 LuxG

The flavin reductase is encoded by *luxG* in the *lux* operon of luminescent marine bacteria. The recombinantly produced enzyme is homodimeric and does not have a prosthetic group after purification. It catalyses the reduction of FMN to $FMNH_2$ using NAD(P)H as a reducing agent, though the preferred substrate is assumed to be NADH. The NADH:oxidoreductase supplies the bioluminescence reaction with reduced FMN. Knockout of the *luxG* gene from *P. leiognathi* TH1 exhibits weaker light emission, therefore the researchers suggest that LuxG is the main source of reduced FMN for the reaction *in-vivo* (55). Since free $FMNH_2$ is prone to fast oxidation in the cell, the transfer of the reduced species to bacterial luciferase was subject to different hypotheses. Research suggests that no LuxG-luciferase complex is built during the transfer, instead reduced FMN is released and supplied to the luciferase via free diffusion (56).

1.5.3 LuxF

The *luxF* gene was first discovered in *P. phosphoreum* in 1988 and was found to have a high degree of sequence homology with *luxB* (ca. 30 %). For this reason, it was suspected that *luxF* has evolved by gene duplication with subsequent deletion of about 100 amino acids. Due to this high similarity and the location of the gene in the *lux* operon, it was assumed that the protein had a structural role in the bioluminescence reaction. Anyway, its function was considered non-essential for light production, as the gene was only found in some *Photobacterium* species and not all luminous bacteria (22). The crystal structure of LuxF was solved by Moore and colleagues. It occurs as a homodimer with monomers folding into a structure similar to $(\beta/\alpha)_8$ barrel proteins, though parts of this fold are missing. Each of the monomer binds two flavin derivatives, more precisely FMN with myristate attached covalently to the C-6 of the flavin isoalloxazine ring (6-(3′-(*R*)-myristyl)-FMN, short myrFMN, Figure 5). Due to the stereoselectivity of this reaction it was suspected that the covalent bond is subject to enzyme catalysis and may even be an unwanted side product generated by the light reaction itself (57, 58).

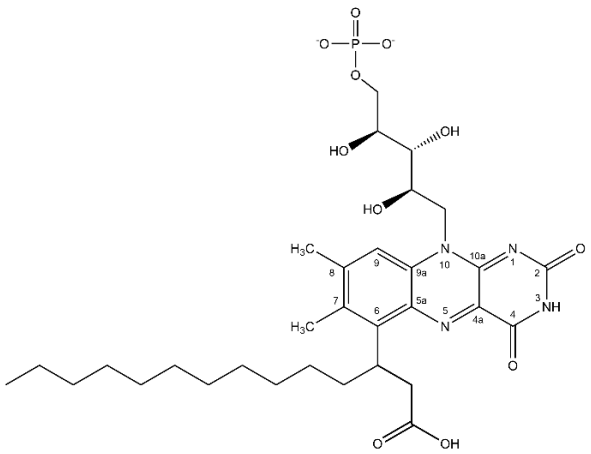

Figure 5: Structure of 6-(3′-(*R*)-myristyl)-FMN (myrFMN).

After the hypothesis that LuxF might act as a scavenger for myrFMN in order to prevent inhibition of the luciferase (58), different approaches were taken to test this theory. The structure of apo-LuxF was solved and confirmed four ligand binding sites. Dissociation constants for myrFMN with LuxF and with luciferase, respectively, revealed that both proteins are able to bind myrFMN. However, binding to LuxF is much tighter, which allows it to scavenge myrFMN (59). These findings were confirmed by an *in-vitro* assay that showed myrFMN was produced during the bioluminescence reaction and inhibits the luciferase. This inhibitory effect can be reduced by providing apo-LuxF which captures the side product. Moreover, a correlation of myrFMN production and light intensity of different bacterial strains was demonstrated (60). In accordance with the above findings, myrFMN has been proven to be a side product of the light reaction not only in *Photobacteria* carrying *luxF*, but also in other bioluminescent marine

bacteria which lack this gene (59, 60). The complete reaction including all structural proteins encoded by the *lux* operon is shown in Figure 6. The scheme illustrates the provision of aldehyde substrate by LuxCDE, regeneration of FMN by LuxG, the enzyme catalysing the actual light reaction, LuxAB, and the optional scavenger of myrFMN, LuxF.

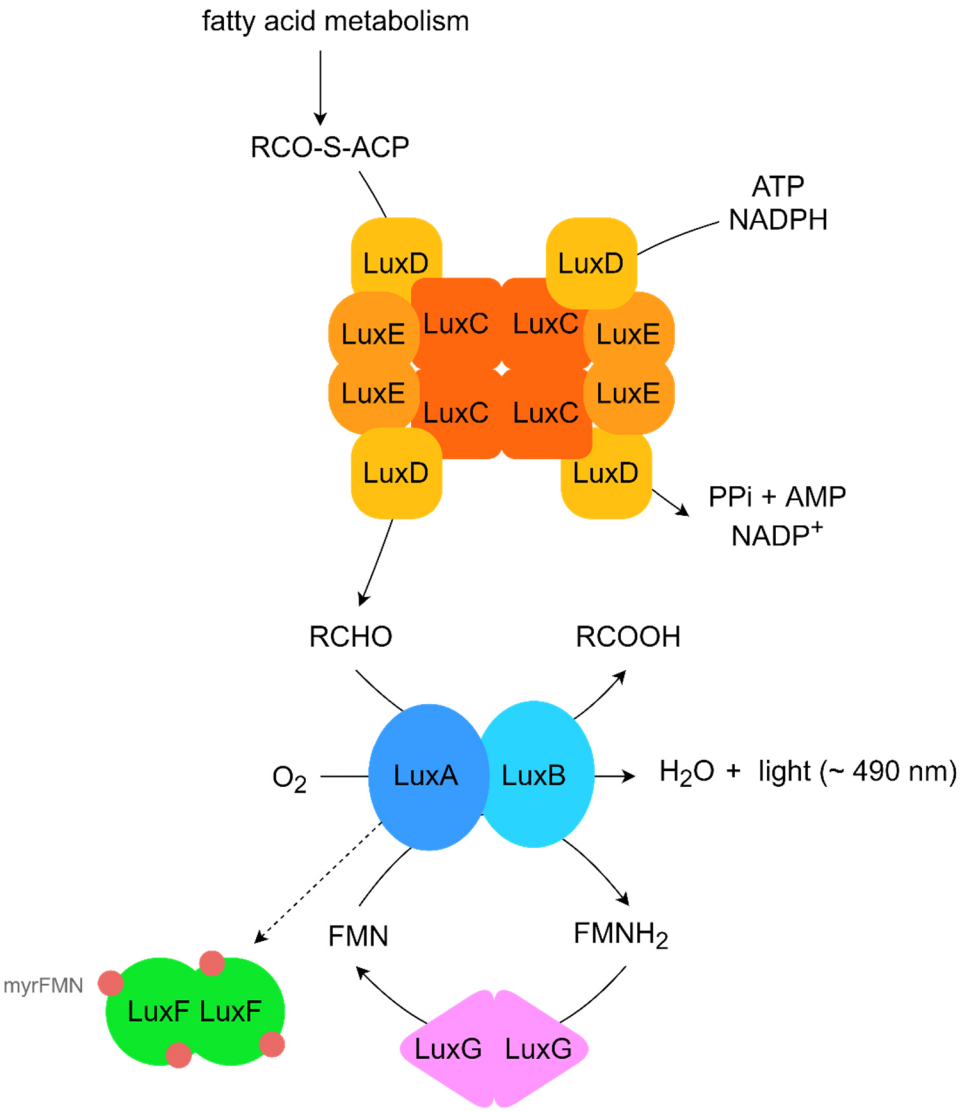

Figure 6: Schematic representation of the bioluminescence reaction completed by the products of the *lux* operon. The LuxCDE (yellow/orange) complex reduces a fatty acid from the cell's metabolism to the corresponding aldehyde and thereby provides part of the substrate to the bacterial luciferase LuxAB (blue/cyan). LuxG (violet) oxidises the reduced FMN in an NADH-dependent reaction (not shown). LuxF (green) is present in some *Photobacterium* species and binds the inhibitor myrFMN (red), which is generated during the bioluminescence reaction.

1.6 Uses in Biotechnology

The range of applications for bacterial luciferase based systems is a fast-growing field, especially as high-throughput assays require fast and easy detection methods. Bioluminescence has been widely used for this purpose. The utilization of bacterial luciferase or the whole *lux* operon to monitor promoter activity has been reported many times (61). With the help of a reporter system it is possible to visualize gene regulation of bacteria during host infection. This was, for example, studied with a virulence gene of *Salmonella enterica*. The *lux* operon was cloned downstream of the native regulatory region for the gene of interest and introduced into a model organism. Expression patterns could then be observed in different organs of the infected host (62).

Another important application of bioluminescence is the monitoring of environmental or food samples regarding contaminations. For this approach, reporter phages specific to the tested bacterial strain are often used. These phages can then transduce the ability to produce bioluminescence to the infected bacterium. Using this method, *Bacillus anthracis* spores can be detected in water samples (63).

1.7 Aim

The aim of this thesis was the recombinant production of the genes responsible for bioluminescence from different marine bacteria. The main focus was on the enzyme catalysing the light reaction, luciferase (LuxAB), and its characterisation by comparing different strains. Furthermore, the effect of LuxF, a putative scavenger of myrFMN, on the intensity of the light produced was studied using an *in-vivo* assay with whole operon recombinantly expressed in *Escherichia coli*.

2 Materials

2.1 General

All commercially available chemicals and media components were obtained from Sigma Aldrich (now Merck, Darmstadt, Germany), Merck, Carl Roth (Karlsruhe, Germany), VWR (Radnor, PA, USA), Fluka by Honeywell International (Morristown, NJ, USA) or Macherey-Nagel (Düren, Germany). Restriction enzymes, polymerases and purification kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA), New England Biolabs (Ipswich, MA, USA) and Promega (Madison, WI, USA). “His-Tag XP Rabbit mAB” and “Anti-rabbit IgG, HRP-linked AB” antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Gerhard Hofer, MSc, from the University of Graz kindly provided a Strep-Tactin HRP conjugate from IBA life sciences (Göttingen, Germany). Primers were ordered from Sigma Aldrich.

2.2 Culture Media

Media components were autoclaved together at 120 °C for 20 min, if not stated otherwise. For plates additionally 15 g/L agar was added.

LB medium:

10 g/L	Peptone/tryptone
5 g/L	NaCl
5 g/L	Yeast extract

Artificial Sea Water:

28.13 g/L	NaCl
0.77 g/L	KCl
1.60 g/L	$CaCl_2$ x 2 H_2O
4.80 g/L	$MgCl_2$ x 6 H_2O
0.11 g/L	$NaHCO_3$
3.50 g/L	$MgSO_4$ x 7 H_2O

Sea water medium:

10 g/L	Yeast extract
10 g/L	Peptone
750 ml/L	Artificial sea water

For sea water medium artificial sea water had to be autoclaved separately and mixed with the rest after cooled to 55 °C.

2.3 Buffers and Solutions

- Strep binding buffer: 20 mM Na_3PO_4, 280 mM NaCl, 6 mM KCl, pH 7.4
- Strep elution buffer: 20 mM Na_3PO_4, 280 mM NaCl, 6 mM KCl, 2.5 mM desthiobiotin, pH 7.4
- Strep regeneration solution: 0.5 M NaOH
- Lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8
- Wash buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8
- Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 8
- Storage buffer: 20 mM Na_3PO_4, 280 mM NaCl, 6 mM KCl, 1 mM DTT, pH 7.4
- YcnD buffer: 20 mM Tris, 100 mM NaCl, pH 8
- Agarose gel electrophoresis TAE-buffer (50x): 2 M Tris/acetate, 0.05 M EDTA, pH 8.5
- DNA Standard: Gene Ruler 1 kb DNA ladder (Thermo Fisher Scientific)

2.3.1 SDS-PAGE Buffers and Gels

Table 1: SDS gel preparation.

	Separating gel 12.5 %:	**Stacking gel 5 %**
40 % acryl amide / 0.8 % Bis	1.56 mL	281.5 µL
1.5 M Tris-HCl, pH 8.8	1.875 mL	-
0.5 M Tris-HCl, pH 6.8	-	312.5
20 % SDS	25 µL	12.5 µL
dH_2O	1.495 mL	1.845 mL
10 % ammonium persulfate	24 µL	12.5 µL
TEMED	5 µL	2.5 µL

- SDS-PAGE running buffer (5x): 15 g/L Tris, 71 g/L Glycine, 2.5 g/L SDS, 1.68 g/L EDTA
- SDS-Page staining solution: 7.5 % acetic acid, 50 % ethanol, 0.25 % Coomassie Brilliant Blue R
- De-staining Solution: 7.5 % acetic acid, 50 % ethanol

- Sample buffer (4x): 63 mM Tris-HCl, 2 %SDS, 0.01 % Bromophenol Blue, 10 % glycerol, pH 6.8
- Sample buffer (2x): 450 µL sample buffer (4x), 450 µL dH_2O, 100 µL DTT (300 mM stock)
- Protein Standard: PageRuler^{TM} prestained (Thermo Fisher Scientific)

2.3.2 Buffers for Western Blot

- Blotting buffer: 25 mM Tris base, 190 mM glycine, 20 % ethanol, pH 8.3
- Tris buffered saline (TBS): 50 mM Tris base, 150 mM NaCl, pH 8
- TTBS: 50 mM Tris base, 150 mM NaCl, 0.2 % Triton-X-100, pH 8
- Blocking solution: 50 mM Tris base, 150 mM NaCl, 0.2 % Triton-X-100, 5 % milk powder
- Clarity Western ECL Substrate (Bio-Rad Laboratories, Inc): peroxide reagent and luminol/enhancer reagent
- Solution for detection of His-tag:
  - 1st antibody (anti-His): His-Tag XP Rabbit mAB 1:12000 dilution in TTBS with 5 % BSA
  - 2nd antibody: Anti-rabbit IgG, HRP-linked AB 1:5000 dilution in TTBS with 5 % milk powder
- Solution for detection of Strep-tag:
  - Strep-Tactin HRP conjugate 1:100000 dilution in TTBS with 5 % BSA

2.3.3 Stock Solutions

- 1 M isopropyl-β-D-thiogalactopyranoside (IPTG)
- 100 mg/mL ampicillin
- 50 mg/mL kanamycin
- 20 mg/mL chloramphenicol
- 100 µg/mL tetracycline
- 250 mg/mL arabinose
- 1 M phenylmethanesulfonyl fluoride (PMSF)
- 1 M dithiothreitol (DTT)

2.4 Kits

- GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific, Inc.)
- Wizard SV Gel and PCR Clean-Up System (Promega GmbH)
- GeneJET Genomic DNA Purification Kit (Thermo Fisher Scientific, Inc.)

2.5 Bacterial Strains

- *E. coli* TOP10: for vector amplification
- *E. coli* BL21 Star (DE3): for expression
- *E. coli* BL21 (DE3) Rosetta™ by Novagen: for expression, chloramphenicol resistance
- *E. coli* BL21 [pG-KJE8] by TaKaRa Bio Inc: for expression, chloramphenicol resistance

Table 2: Bacterial strains used for *luxAB* amplification from genomic DNA.

Strain	Abbreviation	Sequence information	Molecular Mass with tag
Photobacterium leiognathi	TH1	complete	α subunit: 42.8 kDa β subunit: 40.3 kDa
	S1	-	-
	svers1.1	-	-
	ATCC 25521	complete	α subunit: 43.1 kDa β subunit: 40.2 kDa
	ATCC 25587	-	-
Photobacterium mandapamensis	ATCC 27561	complete	α subunit: 42.7 kDa β subunit: 39.8 kDa
Vibrio harveyi	ATCC 14126	complete	α subunit: 42.5 kDa β subunit: 38.8 kDa
	ATCC 33867	-	-
Aliivibrio fischeri	ATCC 7744	complete	α subunit: 42.6 kDa β subunit: 39.8 kDa
	ATCC 14546	-	-
	ATCC 49387	-	-

Table 3: Constructs used for expression.

Host	Plasmid	Genes	Re-sistances[1]	Final AB[2] Concentration
E. coli BL21 [pG-KJE8]	pET51b(+)	*luxAB*_TH1	Amp^R Cm^R	100 µg/mL 20 µg/mL
E. coli BL21 Star (DE3)	pET51b(+)	*luxAB*_25521	Amp^R	100 µg/mL
E. coli BL21 [pG-KJE8]	pET51b(+)	*luxAB*_27561	Amp^R Cm^R	100 µg/mL 20 µg/mL
E. coli BL21 [pG-KJE8]	pET51b(+)	*luxAB*_14126	Amp^R Cm^R	100 µg/mL 20 µg/mL
E. coli BL21 [pG-KJE8]	pET51b(+)	*luxAB*_7744	Amp^R Cm^R	100 µg/mL 20 µg/mL
E. coli BL21 Star (DE3)	pET28a	*luxCDABFEG*_27561	Kan^R	50 µg/mL
E. coli BL21 Star (DE3)	pET28a	*luxCDABEG*_27561	Kan^R	50 µg/mL
E. coli BL21 Star (DE3)	pET28a	*luxCDABFEG*_25521	Kan^R	50 µg/mL
E. coli BL21 Star (DE3)	pET28a	*luxCDABEG*_25521	Kan^R	50 µg/mL
E. coli BL21 (DE3)	pET21a	*ycnd*	Amp^R	100 µg/mL

[1] Amp = ampicillin, Cm = chloramphenicol, Kan = kanamycin
[2] Antibiotics

2.6 Primers

Table 4: Primers for amplification of *luxAB* and subsequent cloning into the pET51b(+) vector.

Strain	Primer Name	Tm [°C]
TH1	forward TH1_pET51b_fwd	60
	reverse TH1_pET51b_rev	59
ATCC 25521	forward 5521_pET51b_fwd	59
	reverse 5521_pET51b_rev	59
ATCC 27561	forward 7561_pET51b_fwd	60
	reverse 7561_pET51b_rev	59
ATCC 14126	forward 4126_pET51b_fwd	62
	reverse 4126_pET51b_rev	63
ATCC 7744	forward 7744_pET51b_fwd	61
	reverse 7744_pET51b_rev	62

2.7 Equipment

PCR Device:	Primus 25 Advanced (PEQLAB)
DNA Electrophoresis:	Sub-Cell GT Electrophoresis Cell (Bio-Rad)
	Gel Doc 2000 (Bio-Rad)
Protein Electrophoresis:	Mini-PROTEAN® Tetra Vertical Cell (Bio-Rad)
Power supply:	PowerPac™ Basic Power Supply (Bio-Rad)
Shaker:	Infors HT Multitron Standard
Centrifuges:	Thermo Scientific™ Sorvall™ RC 6 Plus
	Thermo Scientific™ Heraeus™ Labofuge™ 400R
	Thermo Scientific™ Sorvall™ Legend™ Micro 17R
	Eppendorf® Centrifuge 5810R
Sonication:	Sartorius LABSONIC® P
	Sartorius LABSONIC® L
FPLC:	ÄKTAexplorer 100 (GE Healthcare Life Sciences)
Purification columns:	HisTrap™ HP 5 mL (GE Healthcare Life Sciences)
	StrepTrap™ HP 5 mL (GE Healthcare Life Sciences)
	Superdex® 200 Prep Grade (GE Healthcare Life Sciences)
Various:	Specord 250 (Analytik Jena)
	Centricons: Amicon™ Ultra-15 Centrifugal Filter Units
	Eppendorf® Thermomixer comfort
	ThermoSavant ISS110 Integrated SpeedVac® System
	IKA® Vortex 2
	SensoPlate™ 24 Well Microplate, black, transparent bottom, with lid (Greiner Bio-One)
	PIPETMAN® Classic (Gilson)
	Centro LB 960 (Berthold Technologies)
	CLARIOstar® Plus (BMG Labtech)

3 Methods

3.1 Strep and His tagged Bacterial Luciferases

3.1.1 *luxAB* Preparation

The first step to specifically tagged protein was the amplification of the two genes of the *lux* operon which build the bacterial luciferase. Primers were designed for the five strains of which complete or partial sequence information of *luxA* and *luxB* was available (Table 2). Forward primers carried a restriction site (BamHI-HF or SalI) at the 5'-end followed by the complementary sequence for around 30 bases of *luxA* excluding the start codon ATG. Reverse primers were also built with a restriction site (NotI-HF) and 30 bases complementary to *luxB* but skipping the stop codon (see appendix Table 11). Since the adenine and thymine content of these complementary sequences was rather high, primers had to be designed with an increased number of nucleotides to reach adequate melting temperatures.

Melting and annealing temperatures were calculated with NEB tm calculator for Q5 High-Fidelity DNA Polymerase (NEB). Due to the length of the primers, the actual temperatures used for the extension step of the PCR were determined by performing numerous reactions with varying annealing temperatures. Temperature programs which lead to positive PCR results are listed below (Table 7). The ordered primers were dissolved in ddH_2O according to the technical datasheet and then diluted 1:10. The TH1 primer pair was also used for the bacterial strains S1 and svers1.1, the ATCC 25521 primer pair for the bacterial strains ATCC 25587, ATCC 14126 primers for ATCC 33867 bacterial strain; moreover ATCC 7744 primers were used for the bacterial strains ATCC 14546 and ATCC 49387.

The genes were amplified via polymerase chain reaction (PCR) using isolated genomic DNA of the specific strains as template. The bacteria were grown in 10 mL sea water medium overnight (30 °C, 140 rpm) and DNA was isolated following the protocol for gram-negative bacteria of the genomic DNA purification kit. For *P. leiognathi* ATCC 25521 100 ng of the vector pET28a_*luxCDABEG* was used as a template.

Table 5: PCR mix for *luxAB* amplification.

Components	Volume	Final Concentration/Amount
ddH_2O	11.75 µL	
5X Q5 Reaction Buffer	5 µL	1X
2 mM dNTPs	2.5 µL	200 µM
10 µM forward primer	2 µL	0.8 µM
10 µM forward primer	2 µL	0.8 µM
template DNA	2 µL	100 ng
Q5 High-Fidelity DNA Polymerase	0.25 µL	0.5 U
	25 µL	

The components for each strain were mixed in the given order (Table 5) and transferred to PCR tubes. The temperature program is given in the table below (Table 6).

Table 6: Thermocycling conditions for PCR of *luxAB.*

Step	Temperature	Time	Cycles
initial denaturation	98 °C	2 min	
denaturation	98 °C	10 s	
annealing	see Table 7	30 s	35x
extension	72 °C	60 s	
final extension	72 °C	10 min	
hold	4 °C	forever	

Table 7: Final annealing temperatures used for the amplification of the specific *luxAB* gene and restriction enzymes used for the digest.

Strain	Calculated Annealing T	Used Annealing T	Restriction Enzymes
TH1	60 °C	54 °C	BamHI / NotI
ATCC 25521	60 °C	60 °C	SalI / NotI
ATCC 27561	60 °C	54 °C	BamHI / NotI
ATCC 14126	63 °C	52 °C	BamHI / NotI
ATCC 7744	62 °C	62 °C	BamHI / NotI

Aliquots of 2 µL per reaction were analysed on an agarose gel to confirm amplification of DNA with a length of 2100-2200 bp. DNA obtained from all PCR reactions was purified using Promega's Wizard® SV Gel and PCR Clean-Up System according to the manual for processing of PCR amplifications.

Different approaches were used to digest the PCR products (Table 7). For ATCC 25521 and 25587 SalI and NotI (Thermo Fisher Scientific) were used. The reaction mix was incubated at 37 °C for three hours and then heat inactivated at 80 °C for 20 min. The remaining DNA sequences were digested using BamHI-HF and NotI-HF (NEB), also at 37 °C but for 30 min only. These reactions were purified via Wizard SV Gel and PCR Clean-Up System (Promega GmbH) using the PCR clean-up method.

3.1.2 Agarose Gels

As mentioned above, agarose gels were used to analyse the size of DNA sequences. This was done after DNA amplification and after digestion of DNA using restriction enzymes. Preparative agarose gels, where DNA bands were cut from the gel to be purified, were used for vectors and some PCR products cut for ligation.

For all purposes mentioned, 1 % agarose gels were used. For this, the appropriate amount of agarose was added to TAE buffer (1x) and dissolved by heating. After cooling the solution to about 60 °C 3-5 µL HD Green™ Plus DNA stain (INTAS Science Imaging Instruments) was added to allow UV detection later. As a reference for DNA size determination "Gene Ruler 1 kb DNA ladder" Thermo Fisher Scientific was used.

3.1.3 Chemical Transformation

Transformation of bacteria was used to introduce plasmid DNA to different bacterial strains. The purpose was to multiply the mentioned plasmid DNA, repair nicks that followed ligation or transfer vectors to expression strains for protein production.

Regardless of the chemically competent bacterial cells used for transformation, the heat-shock protocol remained the same. The competent cells were stored at -80 °C until use and then thawed on ice. 1 µL of vector DNA was gently mixed with 100 µL cells and incubated on ice for 15-30 min. Next, the cells were "heat-shocked" at exactly 42 °C for 2 min. After cooling again for 1-5 min 900 mL LB medium was added. The cells were then allowed to recover for 30-60 min at 37 °C and shaking. Afterwards, the tubes were spun at 13300 rpm for 1 min and

the pellet was resuspended in around 100 µL of LB medium. This suspension could then be plated on LB-agar plates containing the appropriate antibiotic(s). The cells grown overnight at 37 °C were then ready for further use.

3.1.4 Vector Preparation

To obtain large amounts of DNA, *E. coli* TOP 10 cells were transformed with the selected vector pET51b(+). A transformant was chosen from the plate to prepare an overnight culture (ONC) in 10 mL LB. The vector was then purified using GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific). Two different restriction digests were carried out. For the first the vector DNA was digested with NotI-HF and BamHI-HF (NEB) at 37 °C for 30 min. The second approach required the restriction enzymes SalI and NotI (Thermo Fisher Scientific). This mix was incubated at 37 °C for three hours.

30-60 min before terminating the reactions alkaline phosphatase was added. The digests were stopped by adding loading dye before separation on an agarose gel. The bands with a length of around 5200 bp were cut from the gel and the DNA was isolated using the Wizard SV Gel and PCR Clean-Up System (Promega GmbH).

3.1.5 Ligation

The digested vector and *luxAB* genes were ligated following standard protocol. A vector to insert ratio of 1:3 was applied in 20 µL reaction setup using T4 DNA ligase and the corresponding buffer. The reactions were incubated at 16 °C overnight. Moreover, a re-ligation control was performed, applying the same protocol but using water instead of insert. The next day all ligation reactions were inactivated by incubating them at 65 °C for 10 min. Afterwards the DNA was transferred to *E. coli* TOP 10 cells using 10 µL of the mix (see section 3.1.3 Chemical Transformation).

3.1.6 Colony PCR

Four to six colonies per transformation plate were picked to confirm successful insertion of *luxAB* into pET51b(+). The colonies were partly plated on a fresh LB-agar plate, partly resuspended in 30 µL ddH_2O. The cell suspensions were heated to 95 °C for 10 min before spinning them at 13300 rpm for 1 min. 8 µL of the supernatant were used for a colony PCR (cPCR) together with 12.5 µL DreamTaq Master Mix (Thermo Fisher Scientific) and 2 µL of the corresponding forward and reverse primers, which were the same as those used for DNA

amplification. For each strain, a positive control reaction with genomic DNA as template was performed. The temperature program for the PCR can be found in the table below (Table 8), annealing temperatures remained unchanged.

Table 8: Thermocycling conditions for cPCR of *luxAB*.

Step	Temperature	Time	Cycles
initial denaturation	95 °C	5 min	
denaturation	95 °C	30 s	
annealing	see Table 7	60 s	30x
extension	72 °C	60 s	
final extension	72 °C	5 min	
hold	4 °C	forever	

The reactions were analysed on an agarose gel to determine clones which, presumably, carried the desired construct. ONCs were prepared to then purify the vectors with GeneJet Plasmid Miniprep Kit (Thermo Fisher Scientific). Vectors were prepared according to company guidelines and sent to Microsynth AG for sequencing.

3.1.7 Expression Tests

Three different *E. coli* based expression strains were tested: *E. coli* BL21 Star (DE3), *E. coli* BL21 (DE3) Rosetta™ and *E. coli* BL21 [pG-KJE8] (TaKaRa). First, the three cell types were transformed with the sequence verified constructs (TH1, ATCC 25521, ATCC 27561, ATCC 14126 and ATCC 7744 in pET51b). Afterwards, 4 mL ONCs with all appropriate antibiotics were prepared and incubated overnight (37 °C, 130 rpm). 1 mL of these ONCs was used to inoculate flasks with 50 mL of main culture. In addition to the antibiotics needed for selection, 10 ng/mL tetracycline and 1 mg/mL arabinose (end concentrations) were added to the TaKaRa cultures to induce expression of the chaperon genes. The main cultures were incubated in a shaker at 37 °C and 160 rpm until an OD_{600} of 0.6-0.8 was reached. When the OD_{600} was high enough, IPTG was added (0.1 mM end concentration) to induce expression. The incubation temperature was changed to 20 °C for overnight expression. The cells were then harvested by centrifugation (4600 rpm, 20 min, 4 °C).

The cell pellets were resuspended in about 2 mL lysis buffer. Cell disruption was achieved by sonicating twice with LABSONIC® P for 2.5 min. The lysate was centrifuged at 13300 rpm for 10 min (4 °C).

3.1.8 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

During the expression tests 1 mL samples of the cell culture were taken prior to induction ("uninduced") and cell harvest ("induced") and concentrated 5-fold. Samples were also collected after sonication ("lysate") and the following centrifugation ("pellet" and "supernatant"). The lysate was diluted 20-fold, the supernatant 2-fold. For the pellet a small quantity was resuspended in 200 µL water by vortexing. Of all prepared samples 10 µL were added to the same amount of sample buffer (2x) and incubated for 10 min at 95 °C. Afterwards, 10 µL of this mix was loaded onto the gel. As a reference for molecular weight estimations 5 µL of PageRuler^{TM} prestained standard (Thermo Fisher Scientific) were applied. Electrophoresis was started at 90 V until the dye reached the separating gel and then continued at 160 V. Lastly, the gels were stained with SDS-PAGE staining solution for a few minutes followed by destaining for one hour or longer.

3.1.9 Large-Scale Expression

Based on the expression tests the most suitable host for protein production was chosen for each construct. The experiments were not continued further for the construct pET51b_*luxAB*_TH1 as the expression tests did not show distinct overexpression. *E. coli* BL21 [pG-KJE8] (TaKaRa) was chosen as an expression host for ATCC 27561, 14126 and 7744. LuxAB from ATCC 25521 was produced in *E. coli* BL21 Star (DE3).

The experiment set-up was similar to the one described in section 3.1.7 Expression Tests. For every construct 15 1-L-flasks were prepared as described below and later combined. ONCs were grown in LB medium with the appropriate antibiotics. 5 mL of those were used to inoculate main cultures of 800 mL LB containing antibiotics and for TaKaRa cultures, additionally 10 ng/µL tetracycline and 1 mg/mL arabinose to induce expression of the chaperone proteins. Cultivation was carried out at 37 °C in a shaker (160 rpm). LuxAB expression was induced after reaching an OD_{600} of 0.6-0.8 by adding IPTG (1 mM end concentration). Protein production was completed overnight at 20 °C and 160 rpm.

The next day cells were harvested by centrifugation (5000 rpm, 10 min, 4 °C) and resuspended in around 50 mL of isotonic NaCl solution (0.9 %). After centrifugation (4600 rpm, 1 h, 4 °C) the supernatant was discarded, and cell pellets were weighed and stored at -20 °C until further use.

3.1.10 LuxAB Purification

To find an ideal procedure for the purification of the different LuxABs four different approaches were tested and compared: Purification over StrepTrap or HisTrap alone or a combination of the two, meaning StrepTrap following HisTrap and vice versa.

The cell pellets (6-8 g) were thawed and resuspended in either lysis buffer or strep binding buffer, depending on which column type was used in the first round of purification. Additionally, DTT and PMSF (1 mM end concentration) were added to all cell suspensions, whereas a His inhibitor cocktail was only added to suspensions with lysis buffer (1 mM end concentration). DTT was also added to all wash and elution buffers in the same concentration. The cells were then lysed via sonication with LABSONIC® P for 5 min under pulsing conditions. The lysate was centrifuged at 18000 rpm (40 min, 4 °C) and the resulting supernatant was carefully filtered before loading onto the column.

Prior to sample loading the columns had to be prepared by washing with five or more column volumes (CV) of deionised water. Then the columns were equilibrated with 5 CV of the appropriate buffer (strep binding buffer or lysis buffer) before loading the filtered protein solution. To remove unspecifically bound proteins the columns were washed with 5-10 CV until no protein was detected in the effluent. This was tested by mixing 10 µL sample with 200 µL Bradford reagent and observing if there was a visible change of colour. The targeted protein was eluted in fractions of 3 mL using (strep) elution buffer. The individual fractions were also tested with Bradford and those containing protein were combined and kept on ice. The pooled samples could then be concentrated to a volume of about 1 mL using centricons with a 30 kDa cut-off. Samples were taken before buffer exchange from strep elution buffer to lysis buffer or elution buffer to strep binding buffer, respectively. The buffers were exchanged by concentrating the solution and then adding the other buffer, repeating both steps three times through. This procedure allowed samples which were purified via StrepTrap at first to be purified via HisTrap in the next step, and vice versa.

After washing and equilibrating the columns again, the combined elution fractions from the first step could be purified a second time, as described above. After the second round of purification, the new elution fractions with protein were again combined and the buffer was exchanged to storage buffer for all samples. SDS-PAGE was performed for all collected samples: lysate, pellet after lysis and centrifugation, supernatant, flow through, wash and of course elution fractions. After analysis of the gels it was assumed that the best results were achieved using only one step of StrepTrap column purification. To verify this presumption MALDI-TOF analysis of the elution fractions as well as Western Blots were performed.

In order to receive large amounts of protein, large-scale expressions and purifications via StrepTrap were performed. Expression resulted in pellets with 30-40 g, which were resuspended in about 30 mL strep binding puffer. To improve lysis the cell suspensions where stirred with a spatula tip of lysozyme for 30 min at 4 °C. The cells were then lysed via sonication with LABSONIC® P three times 5 min each with cooling breaks in between. Purification itself was performed as described above. The concentrated and buffer exchanged elution fractions were shock frozen in liquid nitrogen and stored at -20 °C. These large-scale purifications were also analysed via SDS-PAGE, MALDI-TOF and Western Blot.

3.1.11 Western Blot

Western Blot analysis was conducted to provide evidence for the expression and purification of heterodimeric luciferase. Samples were taken from the purification tests of all four luciferases: pellet, supernatant, flow through, and one elution fraction. Moreover, two proteins with either His-tag or Strep-tag were used as positive controls for the detection. The immunoassay following the blot was performed using two approaches, one with anti-His antibody to detect the His-tag on LuxB, the other with Strep-Tactin to detect the Strep-tag on LuxA. For this reason, two gels with the exact same samples were prepared to receive two identical membranes after blotting. Nitrocellulose membranes were cut to the right size and together with the gel, sponges and filter paper arranged as usual for blotting in a chamber. The chamber was filled with blotting buffer (see 2.3.2 Buffers for Western Blot) and run for one hour at 200 mA.

To examine successful blotting, the proteins on the membrane were stained reversibly with PonceauS for a few seconds, before washing with water until protein bands were visible. The membrane was then shaken in blocking solution for one hour and then washed three times with TTBS for 10 min. Afterwards one membrane of the two duplicates was incubated overnight with the 1st antibody (anti-His) solution (4 °C) and the other with the Strep-Tactin conjugate solution for one hour at 4 °C (see 2.3.2 Buffers for Western Blot). The first solution was then removed, and the membranes were washed as explained above. For the detection of the His-tag the membrane had to be incubated with the 2nd antibody solution for one hour (4 °C) followed by another washing step. For detection the two Clarity Western ECL Substrates were mixed 1:1 and poured onto the membrane. After incubation for 3 min the light emitted following the reaction of the substrate with the antibody-linked peroxidase could be detected using a film.

3.1.12 YcnD Purification

The flavin-containing oxidoreductase YcnD, which was needed for the luciferase activity assay, was expressed in *E. coli* BL21 (DE3) cells harbouring the vector pET21a with the His-tagged protein. Protein production was carried out in 14 flasks containing 800 mL LB medium and ampicillin, similarly as described in section 3.1.9 Large-Scale Expression. After harvesting the cells, they were resuspended in lysis buffer with a spatula tip of FMN. The protein was purified via a HisTrap column (see 3.1.10 LuxAB Purification) and the main elution fractions were combined.

The buffer was exchanged to YcnD buffer by dialysis. Therefore, a few centimetres of dialysis tube were prepared by soaking in water. Afterwards, one end of the tube was knotted, and the protein solution was filled in at the other end. Then the other end of the tube was knotted leaving enough space for possible volume expansion. The tube was fixed to a measuring cylinder which was filled with YcnD buffer and left at 4 °C overnight with stirring. The next day the protein concentration was determined using a photometer. The absorption at 450 nm was measured and with the help of the molar extinction factor the concentration could be calculated. Afterwards YcnD was shock frozen in droplets in liquid nitrogen and stored at -20 °C.

3.1.13 Luciferase Activity Assay

To investigate the activity of the different luciferases a plate reader assay was performed. Each luciferase variant was measured in triplicates in black 96-well plates. The detection method was specific to the instrument (luminometer) used, which measured light intensity produced by the bioluminescent reaction. The duration of the measurement was set to 60 s after test runs had shown that after this period the reaction would be finished. All components were prepared in water or strep elution buffer, except for tetradecanal, which was diluted in ethanol. The mix without the substrate present served as a reference measurement (blank).

All parts of the reaction mix (Table 9) were kept on ice at all times and mixed together shortly before the measurement, aside from NADPH. The reaction was executed at room temperature and to start it NADPH was added directly by injection through the plate reader. The light intensity was observed over the course of 60 s.

Table 9: Reaction composition for the luciferase activity assay.

Component	Stock Concentration	Final Concentration	Volume
luciferase	21.1-35.6 µM	400 nM	2.8-4.7 µL
FMN	20 µM	600 nM	7.5 µL
YcnD	16.3 µM	600 nM	9.2 µL
tetradecanal	120 µM	600 nM	1.25 µL
storage buffer	-	-	fill up to 240 µL
NADPH	12.5 µM	500 nM	10 µL

3.1.14 Size Exclusion Chromatography (SEC)

Fast protein liquid chromatography (FPLC) was performed for analytical reasons to find out if the purified protein was present mainly in monomers, dimers or aggregates. The column (Superdex® 200 Prep Grade) was washed with water and equilibrated with strep binding buffer. The samples (1 mL of 1 mg protein /mL) were loaded onto the column and collected in 1 mL fractions after SEC. For each luciferase, fractions of the same size were combined. Those which were thought to be monomer or dimer were concentrated and stored separately at 4 °C overnight, to see if they would form aggregates. One dimer sample was also shock frozen in liquid nitrogen after SEC. To see if storage overnight or shock freezing had any effect on molecular weight distribution, another FPLC run was performed, but sample concentrations were very low at this point. SDS-gel electrophoresis was conducted for chosen samples.

3.2 Light intensity in dependence of LuxF

This assay was performed to gain information on the role of the protein LuxF, encoded by the *luxF* gene, regarding light intensity during the reaction catalysed by the luciferase. Both the whole *lux* operon *luxCDABFEG* and the same operon missing *luxF* (*luxCDABEG*) of two different strains were cloned into the pET28a vector and transferred into *E. coli* (Table 3). These constructs were then grown overnight at 37 °C on LB plates. The next day cultures of 4 mL LB with kanamycin were inoculated with a single colony. The cultures were then cultivated for 4-6 h (30 °C, 140 rpm).

The OD_{650} was measured and all samples were diluted to an OD_{650} of 0.1 in 1 mL LB with the appropriate antibiotic. These dilutions were prepared in 24-well microplates (black with clear bottom and lid), but only the two outermost columns were used (four wells per side), the rest was filled with 1 mL LB, of which the two inner columns served as blanks. 1 mM IPTG was added from the beginning to induce the expression. In the same way cultures of *E. coli* BL21 Star (DE3) carrying pET28a or no plasmid at all (reference measurements) were incubated. *E. coli* without a vector present did not require addition of kanamycin. The plates were then incubated for 10 h (28 °C, 300 rpm). The light intensity and optical cell density at 650 nm (OD_{650}) were measured every 10 min with the help of a script developed for this specific method (see 8 Appendix Figure 26). All samples, including the references, were analysed in four biological and three technical replicates.

4 Results

4.1 Strep and His tagged Bacterial Luciferases

4.1.1 Cloning

The eleven strains depicted in Table 2 were selected for cloning of the two luciferase genes *luxA* and *luxB*. The episomally replicating vector pET51b(+) was chosen for the construct, as it carried a Strep-tagII upstream and a deca-His-tag downstream of the multiple cloning site. This site was under the control of a T7 promoter, so the use of a DE3-genotype strain allowed for induction with IPTG. The vector also carried a gene encoding for β-lactamase to make selection in ampicillin containing medium possible. Due to the lack of sequence information for most of the 11 strains, the five designed primer pairs were also used for related strains. Only genes of which sequence information was available could be successfully amplified and cloned into the vector (Figure 7). The resulting PCR product had a length of about 2200 bp which corresponded to the actual gene size of approximately 2175 bp.

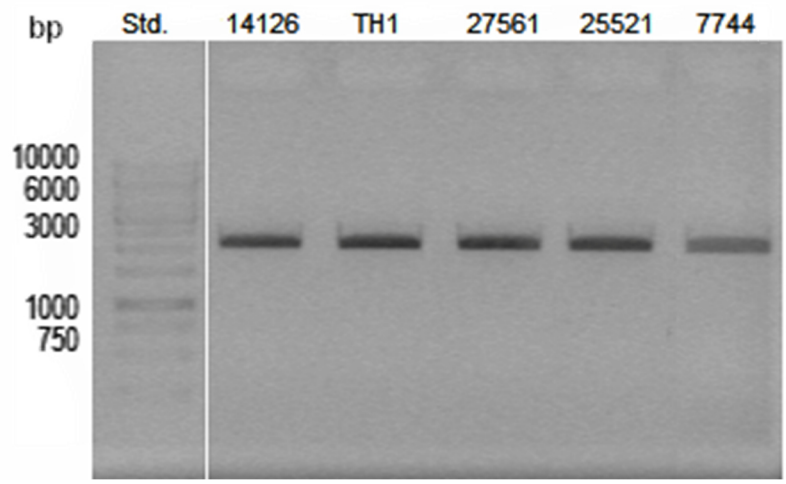

Figure 7: Result of the DNA amplification via PCR.

After transformation of the cells with the ligation mix a cPCR was conducted as a first indication for successful insertion of the genes into the vector. One transformant with a resulting band at the same length as the positive control was chosen for sequencing. Figure 8 shows the cPCR of *luxAB*_14126 and is exemplary for all strains. The remaining results of cPCR can be found in the appendix (Figure 27).

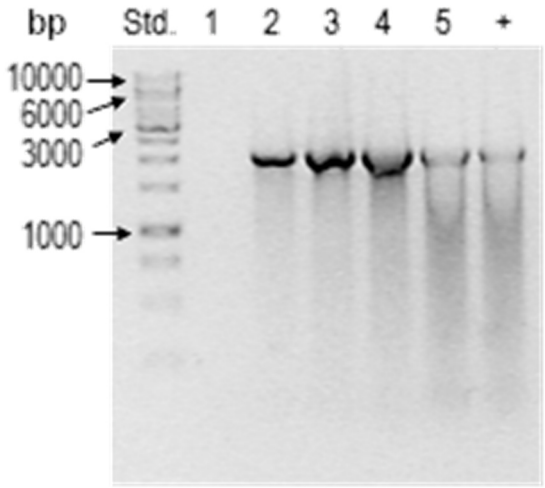

Figure 8: Result of a cPCR of transformants of pET51b_*luxAB*_14126. The numbers 1-5 correspond to the different clones picked from the plate, + is the positive control with genomic DNA as template.

Cloning resulted in five constructs of different luciferases with a Strep-tag on the 5'-end of *luxA* and a deca-His-tag on the 3'-end of *luxB*. An example of the complete construct is shown in Figure 9, the rest of the plasmid maps can be found in the appendix (Figure 28). The sequence-verified vectors (compare Table 3) were then transferred to *E. coli* expression hosts to further use them for protein production.

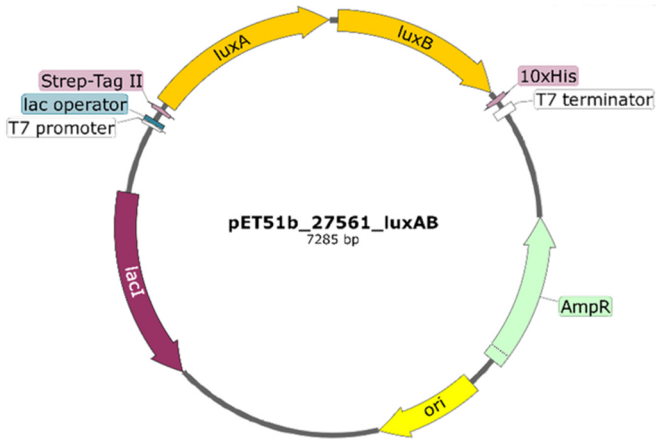

Figure 9: Vector construct of pET51b(+) with *luxA* and *luxB* between the two tags. The vector carried a β-lactamase for ampicillin resistance (AmpR), lac repressor (lacI) and a multiple cloning site regulated by a T7 promoter region.

4.1.2 Protein Biosynthesis

LuxAB expression of the strains TH1, ATCC 27561, 7744, 14126 was tested to find a favourable host for protein production. Three possible *E. coli* expression strains were tried out in small-scale experiments: *E. coli* BL21 Star, *E. coli* BL21 Rosetta™ and *E. coli* BL21 [pG-KJE8]

(short TaKaRa). Samples were drawn as described before (see 3.1.7 Expression Tests) and analysed via SDS-PAGE. It was possible to obtain gels with a clearly detectable over-produced protein at the correct masses of 39 to 43 kDa (indicated with arrows on the following figures). For most luciferases, this was best achieved with the TaKaRa strain (Figure 10, Figure 11).

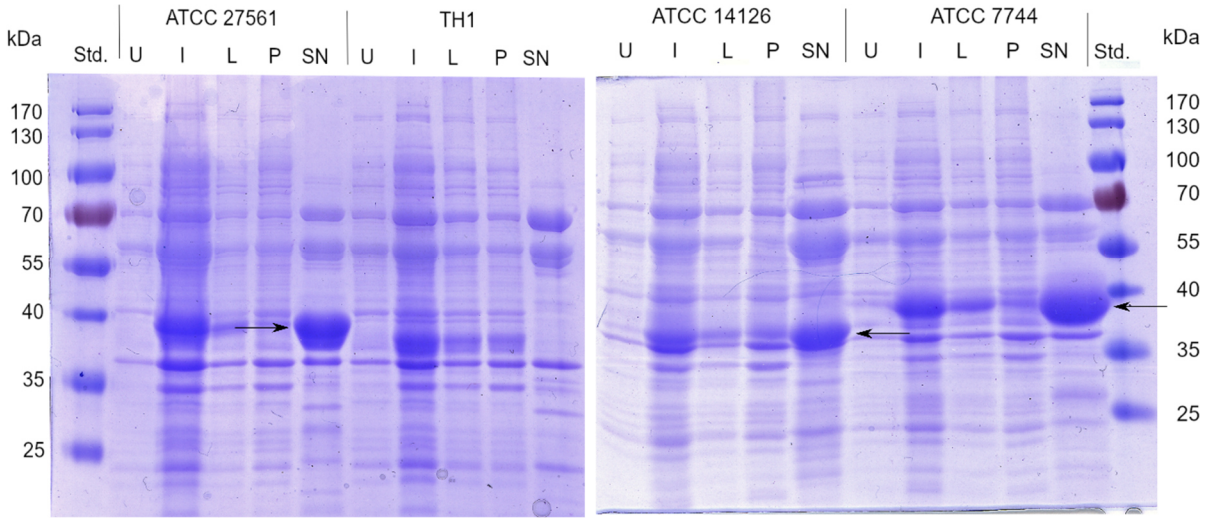

Figure 10: Expression tests in TaKaRa cells. Uninduced (U), induced (I), lysate (L) pellet (P) and supernatant (SN) samples were analysed. Protein samples from the four strains TH1, ATCC 27561, 14126 and 7744 are shown.

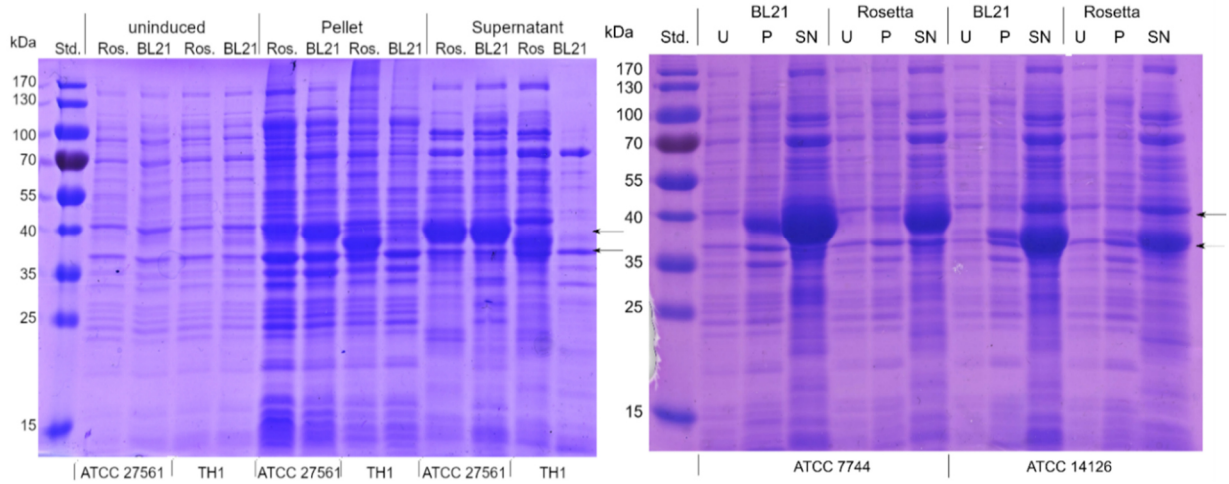

Figure 11: Results of the first round of expression tests. Previously described samples were taken throughout the fermentation process of Rosetta (Ros) and *E. coli* BL21 Star (BL21) cells. Uninduced (U), pellet (P) and supernatant (SN) samples were analysed and the different strains were compared.

Expression of TH1's and ATCC 25521's *luxAB* in TaKaRa was rather low, compared to the other strains (Figure 10, Figure 12). Only overexpression of genes resulting in soluble proteins of a molecular weight between 60 and 70 kDa could be observed. Generally, expression levels of ATCC 25521 *luxAB* were lower than the rest, but *E. coli* BL21 Star resulted in protein bands at the expected position on the gel. The luciferase of TH1 could be produced

sufficiently in Rosetta, but cell growth was affected negatively by the expression and cultivation took much longer than for the other strains. For this reason, the experiment for TH1 luciferase was discontinued.

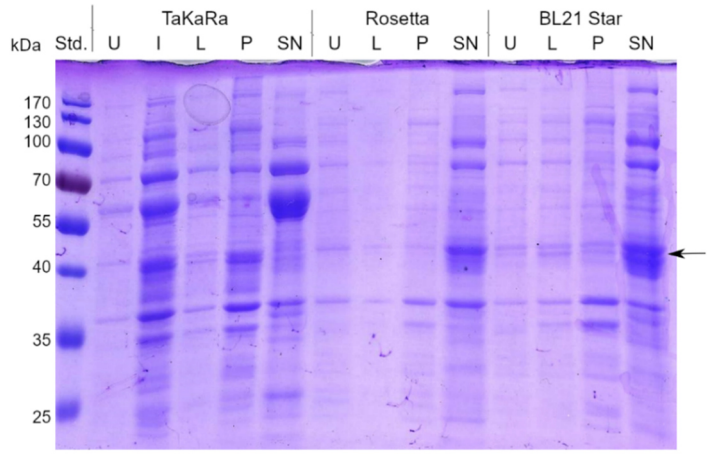

Figure 12: Expression test of luciferase ATCC 25521. Uninduced (U), lysate (L), pellet (P), supernatant (SN) samples were drawn from all three approaches, induced (I) sample only from TaKaRa expression host.

In summary, it can be stated that *E. coli* BL21 [pG-KJE8] (TaKaRa) was the most promising expression host for most strains. The luciferases from ATCC 27561, ATCC 14126 and ATCC 7744 were produced in TaKaRa from this point on. For the luciferase of ATCC 25521, *E. coli* BL21 Star was chosen for further protein production.

4.1.3 Protein Purification

As the recombinantly produced heterodimeric protein was equipped with two different tags, various purification approaches had to be tested. The results of these purification experiments were analysed with SDS-PAGE, MALDI-TOF and Western Blot with an immunoassay directed against the tags on the luciferases.

Starting with SDS-PAGE, one to two protein bands at around 40 kDa were detected in lysate and supernatant (after centrifugation of the lysate) for all luciferases, which was expected. However, part of the protein was still present in all pellet samples. For purification via StrepTrap low amounts of protein were lost by not or poorly binding to the column. Generally, protein quantities obtained with the StrepTrap column seemed to be much lower than with HisTrap. In all tests, the protein purified via HisTrap before StrepTrap was at least partially lost in the flow through and wash fractions of the second purification step, seemingly not binding to the column. Except for the luciferase of ATCC 14126 no protein was detectable in these elution fractions (Figure 13).

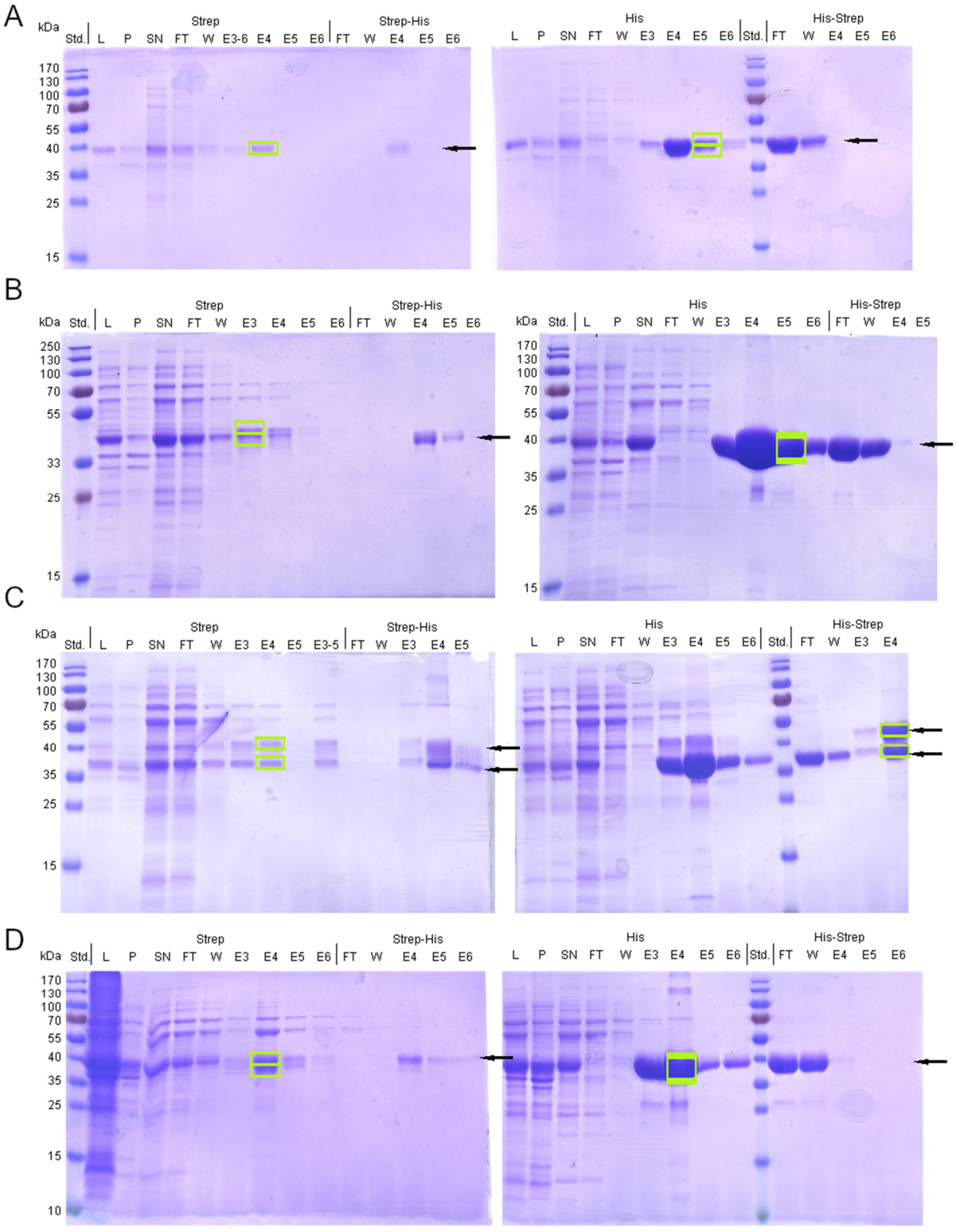

Figure 13: SDS-PAGE analysis of the purification tests. The protein solutions were purified via a StrepTrap column before concentrating the elution fractions and purifying them via HisTrap (A-D, left). The same procedure was done vice versa, starting with HisTrap column purification (A-D, right). The relevant protein bands are marked with arrows. Yellow rectangles indicate bands which were cut out of the gel for MALDI-TOF analysis. **A:** ATCC 25521; **B:** ATCC 27561; **C:** ATCC 14126; **D:** ATCC 7744. L = lysate, P = pellet after cell lysis, SN = supernatant after cell lysis, FT = flow through, W = wash, E = elution fraction.

For the Western Blot, detection via the anti-His antibody often led to smears or additional signals at a higher molecular weight, especially if the protein concentration in the sample was higher. Positive controls for both detection methods showed a signal at the expected sizes of 29 kDa for the protein carrying a Strep-tag (except for ATCC 7744, see Figure 17) and 28.7 kDa for His-tagged YcnD (Figure 14 – Figure 17, panel B). The Strep-tagged positive control was kindly provided by Gerhard Hofer, MSc, from the University of Graz. Unfortunately, more information on the protein was not obtainable.

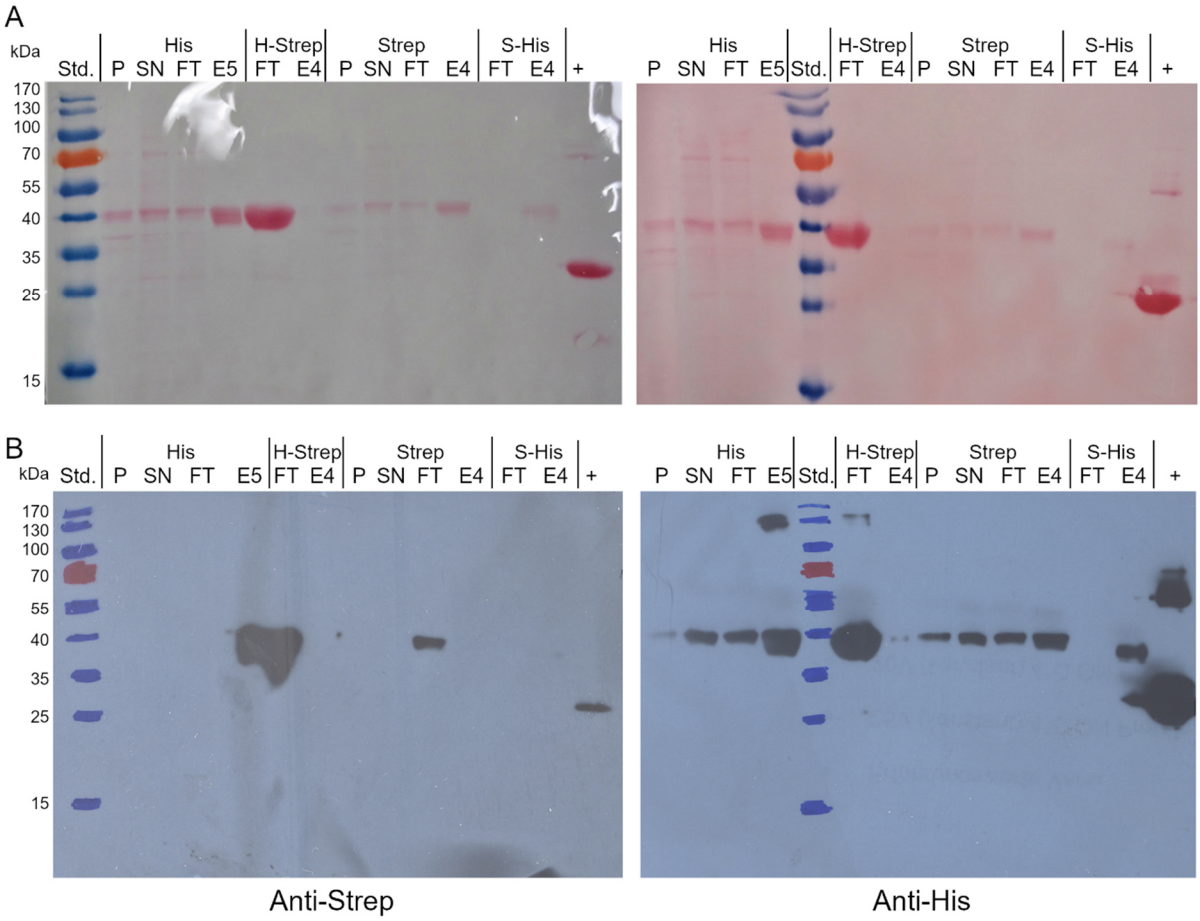

Figure 14: PonceauS staining and immunological analysis of *P. leiognathi* ATCC 25521. Selected samples were applied to gels after the purification tests. Samples from these purification tests were chosen and analysed via Western Blot and immunoassays using different antibodies. **A:** Nitrocellulose membrane after reversible protein staining with PonceauS. **B:** Films after immunological detection of the same membranes. On the left Strep-Tactin was used, which was able to bind to the Strep tag. On the right an antibody directed against the His-tag was utilized. L = lysate, P = pellet after cell lysis, SN = supernatant after cell lysis, FT = flow through, W = wash, E = elution fraction, + = positive control.

The Western Blot confirmed the first assumptions drawn from the SDS-PAGE analysis. For *P. leiognathi* ATCC 25521 (Figure 14) no Strep-tag could be detected in the pellet and supernatant samples, although PonceauS staining clearly showed that the protein transfer onto the membrane was successful. Luciferase α-subunit was detected in the flow through fractions of His-Strep and Strep purification. For purification via HisTrap alone, the anti-His antibody

detection produced a signal at the expected molecular mass, confirming the purification of β-subunit. HisTrap purification followed by StrepTrap gave strong signals in the flow through, affirming the belief that in the second round no protein was bound to the column. The picture is different for tests starting with StrepTrap columns, where after the second purification step, the His-tag carrying β-subunit could still be detected.

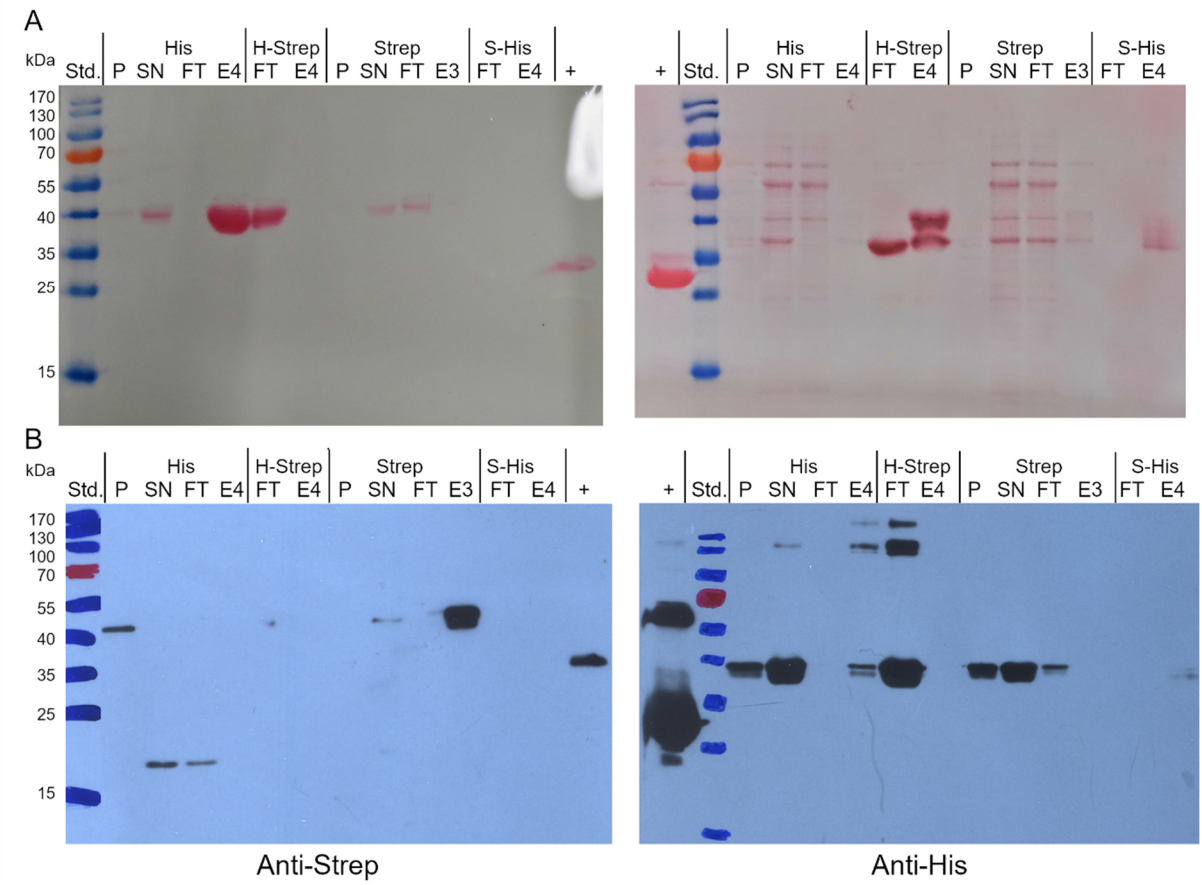

Figure 15: PonceauS staining and immunological analysis of *P. mandapamensis* ATCC 27561. A: Nitrocellulose membrane after reversible protein staining with PonceauS. **B:** Films after immunological detection of the same membranes. On the left Strep-Tactin was used, which was able to bind to the Strep tag. On the right an antibody directed against the His-tag was utilized. L = lysate, P = pellet after cell lysis, SN = supernatant after cell lysis, FT = flow through, W = wash, E = elution fraction, + = positive control.

Analysis of the immunoblot of the luciferase from *P. mandapamensis* ATCC 27561 showed His-detection in the flow through samples of the His-Strep purification and no more signal in the elution fraction (Figure 15). This had already been expected from analysing the polyacrylamide gels and confirmed that purification via HisTrap led to enrichment of β-subunit, which was then again lost in the flow through after StrepTrap purification. The PonceauS stain suggested that for most samples very low protein quantities were blotted onto the “Strep” membrane, which might have led to false negatives. Anyway, even the rather strong elution fraction after His purification did not produce a signal for Anti-Strep which suggests that there really was no or little LuxA, but only LuxB.

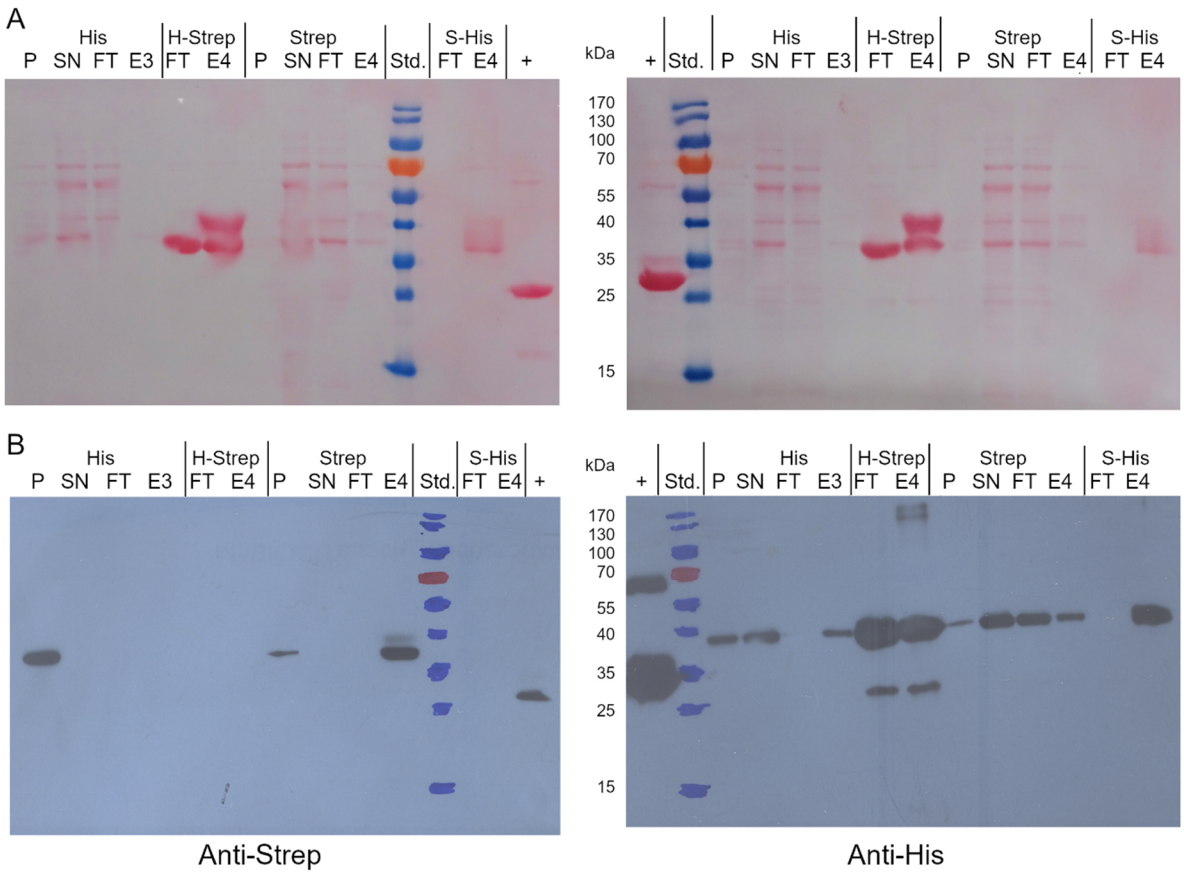

Figure 16: PonceauS staining and immunological analysis of *V. harveyi* ATCC 14126. A: Nitrocellulose membrane after reversible protein staining with PonceauS. **B:** Films after immunological detection of the same membranes. On the left Strep-Tactin was used, which was able to bind to the Strep tag. On the right an antibody directed against the His-tag was utilized. L = lysate, P = pellet after cell lysis, SN = supernatant after cell lysis, FT = flow through, W = wash, E = elution fraction, + = positive control.

The immunological assay of ATCC 14126 also showed that purification via StrepTrap resulted in elution fractions with both α- and β-subunit (Figure 16). All other purification tactics lead to the enrichment of β-subunit. This also applied to approaches where two bands could be seen on the stained gel or membrane and were thought to represent both subunits, because of their difference in molecular weight.

The analysis of the purification tests for ATCC 7744 led to similar conclusions as mentioned above (Figure 17). With this luciferase the α-subunit was also found in elution fractions after purification via HisTrap.

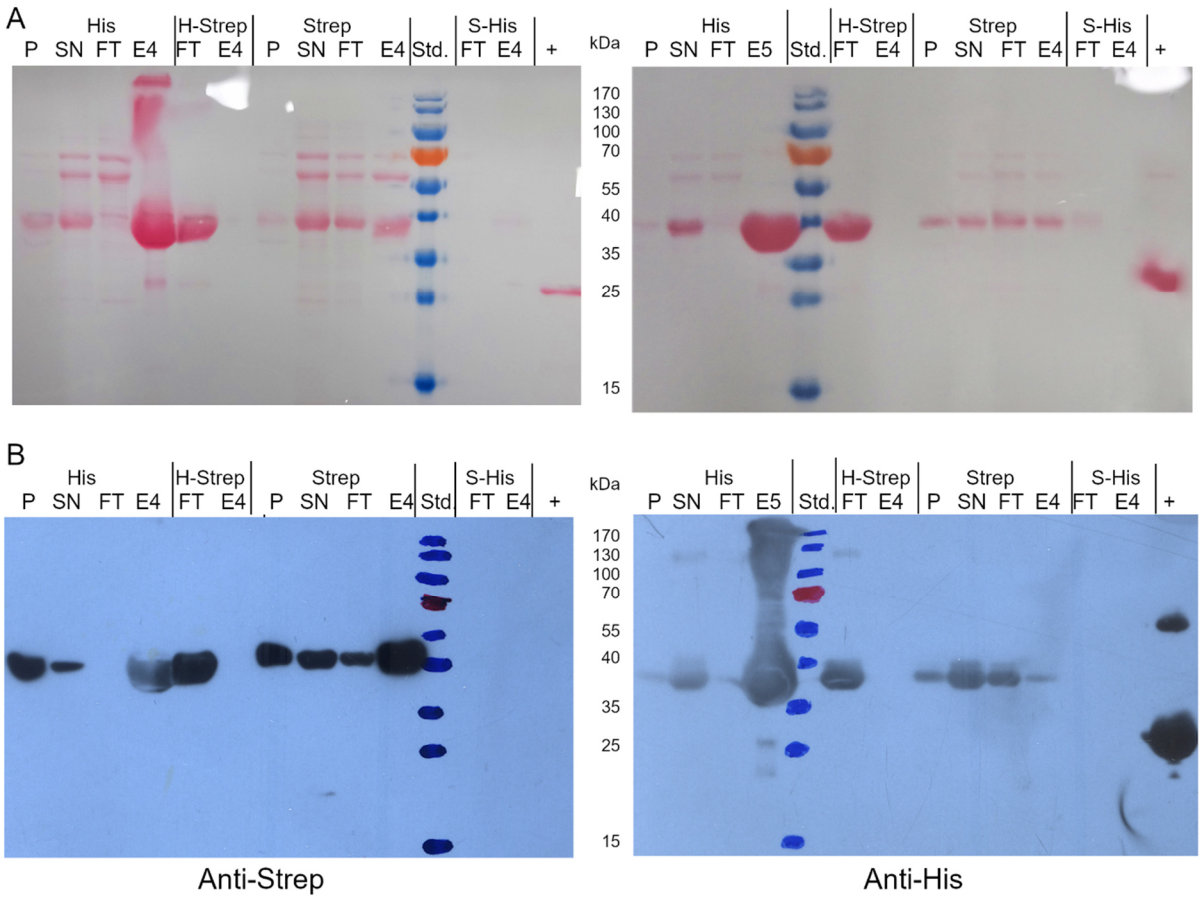

Figure 17: PonceauS staining and immunological analysis of *A. fischeri* ATCC 7744. A: Nitrocellulose membrane after reversible protein staining with PonceauS. **B:** Films after immunological detection of the same membranes. On the left Strep-Tactin was used, which was able to bind to the Strep tag. On the right an antibody directed against the His-tag was utilized. L = lysate, P = pellet after cell lysis, SN = supernatant after cell lysis, FT = flow through, W = wash, E = elution fraction, + = positive control.

Selected samples were cut from the SDS-gel and analysed with MALDI-TOF mass spectrometry (Figure 13, yellow rectangles). The evaluation was done by comparing the data using MassLynx with the theoretical peptide masses obtained by Expasy Peptide Mass. This analysis showed that elution samples after StrepTrap contained both subunits, whereas purification via HisTrap led to β-subunit only. In all samples where two distinct bands were visible in the "Strep" elution fractions, the upper band represented α-subunit, whereas the lower band was β-subunit, as expected. These observations were true for all samples except for ATCC 14126. This luciferase had the biggest molecular weight difference between the two subunits (3.7 kDa), which could also be seen on the SDS-gels. The band at a higher molecular weight could be assigned to α-subunit and the lower to β-subunit not only after Strep but also after His-Strep purification. For this reason, it would have been possible to use two purification strategies for the luciferase from ATCC 14126.

Based on the above data, it was expected that the best results would be reached by purification over StrepTrap column only. To be sure, the experiments were repeated at a larger scale. As

before, SDS-PAGE analysis showed one to two distinct bands, depending on the luciferase variant (Figure 18, right). Moreover, the Western Blot of the purified and buffer-exchanged protein samples indicated that both luciferase subunits were present in all samples. However, a difference could be observed compared to the purification tests: Detection of the Strep-tag with Strep-Tactin showed protein bands at a low molecular weight of around 17-20 kDa (Figure 18, left).

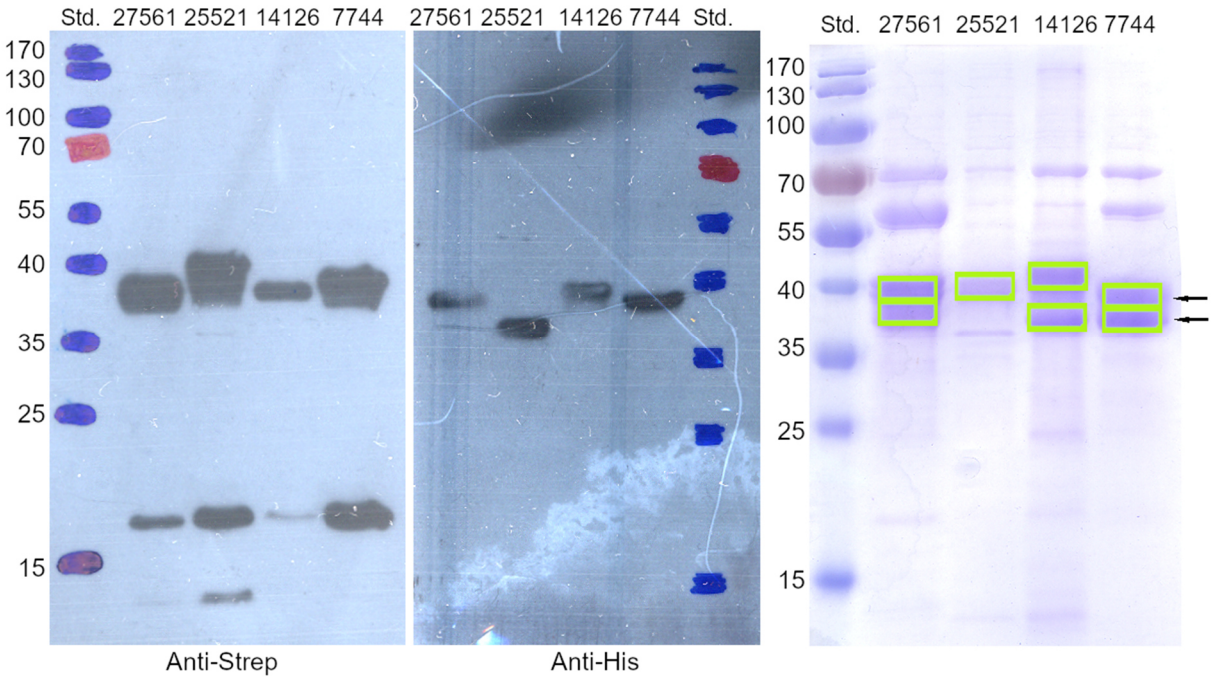

Figure 18: Western Blots (left and middle) and SDS-PAGE analysis (right) of all four luciferases (ATCC 27561, 25521, 14126, 7744) after large-scale purification. Left: Purified luciferase variants of the four strains were analysed using Strep-Tactin antibody against Strep-tag. **Middle:** Purified luciferase variants of the four strains were analysed using antibody against His-tag. **Right:** All lanes show the purified protein after one round of StrepTrap purification. Yellow squares indicate samples cut out for MALDI-TOF analysis.

After SDS-PAGE analysis, samples were cut from the gel again for analysis with MALDI-TOF (Figure 18, yellow squares on the right). For all luciferases, the two subunits were found after purification via StrepTrap column. Both α- and β-subunit could be detected in all samples, regardless of the band seen on the gel, except for ATCC 14126. The bands of this luciferase were clearly separated into both subunits, as explained before. For the rest it was observed that the upper bands were mostly LuxA whereas the lower bands were LuxB, primarily. However, both contained the other subunit, but at a much lower signal strength.

Regarding the protein yield, it can be noted that very low protein concentrations were achieved (Table 10). This could be, at least partially, due to loss of protein during the purification in the flow through and wash fractions.

Table 10: Protein yields after large-scale purification.

Luciferase	Protein Concentration	Protein yield
P. leiognathi ATCC 25521	23.4 µM	0.11 mg/g_{pellet}
P. mandapamensis ATCC 27561	21.1 µM	0.04 mg/g_{pellet}
V. harveyi ATCC 14126	26.2 µM	0.13 mg/g_{pellet}
A. fischeri ATCC 7744	35.6 µM	0.34 mg/g_{pellet}

4.1.4 Luciferase Activity Assay

An assay previously described by Brodl et al. was performed to determine and compare the activities of the purified luciferases (19). For the reaction set-up, a NAD(P)H:FMN oxidoreductase was needed, therefore YcnD was expressed and purified as described before. The concentration was determined spectrophotometrically at 450 nm, which is specific to the FMN cofactor. Knowing the extinction coefficient of the protein (ε_{450} = 12190 $M^{-1}cm^{-1}$), the YcnD concentration was calculated to be 0.326 mM and the protein yield 4.3 mg/g_{Pellet}.

The time course of light emission of the four luciferases that could be purified was measured in triplicates. The activity assay is based on the light reaction catalysed by bacterial luciferase, using a long-chain aliphatic aldehyde as substrate, in this case tetradecanal. The aldehyde is converted to the corresponding fatty acid in an $FMNH_2$ and O_2 dependent reaction, in which light is produced (Figure 19).

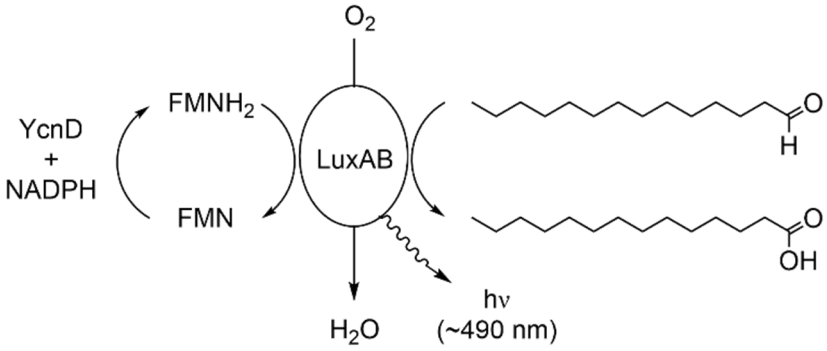

Figure 19: Reaction scheme of the luciferase activity assay. Tetradecanal is oxygenized to myristic acid by luciferase (LuxAB) under utilization of reduced FMN ($FMNH_2$). Light with an emission maximum of 490 nm is produced and detected by the luminometer. The oxidized flavin is then recycled with an NAD(P)H:FMN oxidoreductase (YcnD).

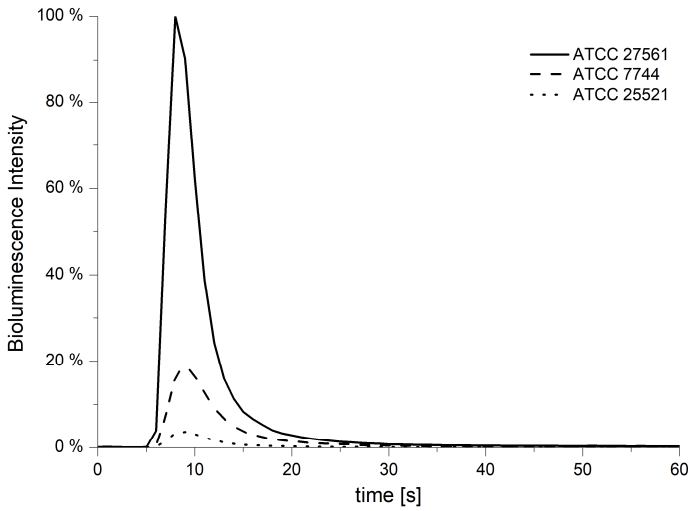

Figure 20: Time course of the light emission during the activity assay. The light intensity was measured for 60 s. The highest signal was obtained for ATCC 27561 and therefore set as 100 % to allow for clear comparison of the luciferases.

Three of the tested recombinant enzymes showed clear activity (Figure 20) whereas the luciferase of *V. harveyi* ATCC 14126 did not produce any light emission and therefore was not plotted in the graph. In all approaches the maximum light intensity was reached after 8-9 s. The highest emission rates were achieved with the luciferase of *P. mandapamensis* ATCC 27561, this value was set as 100 % to be better comparable to the other strains. The other two enzymes hit 20 % (*A. fischeri* ATCC 7744) or 4 % (*P. leiognathi* ATCC 25521), respectively. As the Brodl et al. paper analysed a different set of strains than described here, it was not possible to draw valid conclusions from this comparison. What was noticeable was that *P. leiognathi* exhibited the least intense signal whereas in the mentioned publication a luciferase of another *P. leiognathi* strain (S1) was the one with the highest bioluminescence intensity.

4.1.5 SEC Analysis

Analytical size exclusion chromatography (SEC) was performed for further analysis of the luciferases. It was of particular interest, if the difference in activity could be explained by the occurrence of aggregates or degraded protein. The corresponding chromatograms differed quite clearly (Figure 21). Anyway, the first peak (panel A-D, peak 1) after around 8 mL produced the strongest signal for all luciferases. This peak often containing an additional shoulder indicated the strong presence of aggregates with a molecular weight of about 1600 kDa. Peak No. 2 eluted at 14.3 mL (76 kDa), which roughly fit the molecular weight of protein dimer of 80 kDa, though it was unknown which monomers it consisted of. The third peak at 16.2 mL (35 kDa) roughly corresponded to protein monomer of either α- or β-subunit. Peak 4 and 5

after 22.3 mL and 27 mL showed very low molecular weight peptides, indicating protein degradation.

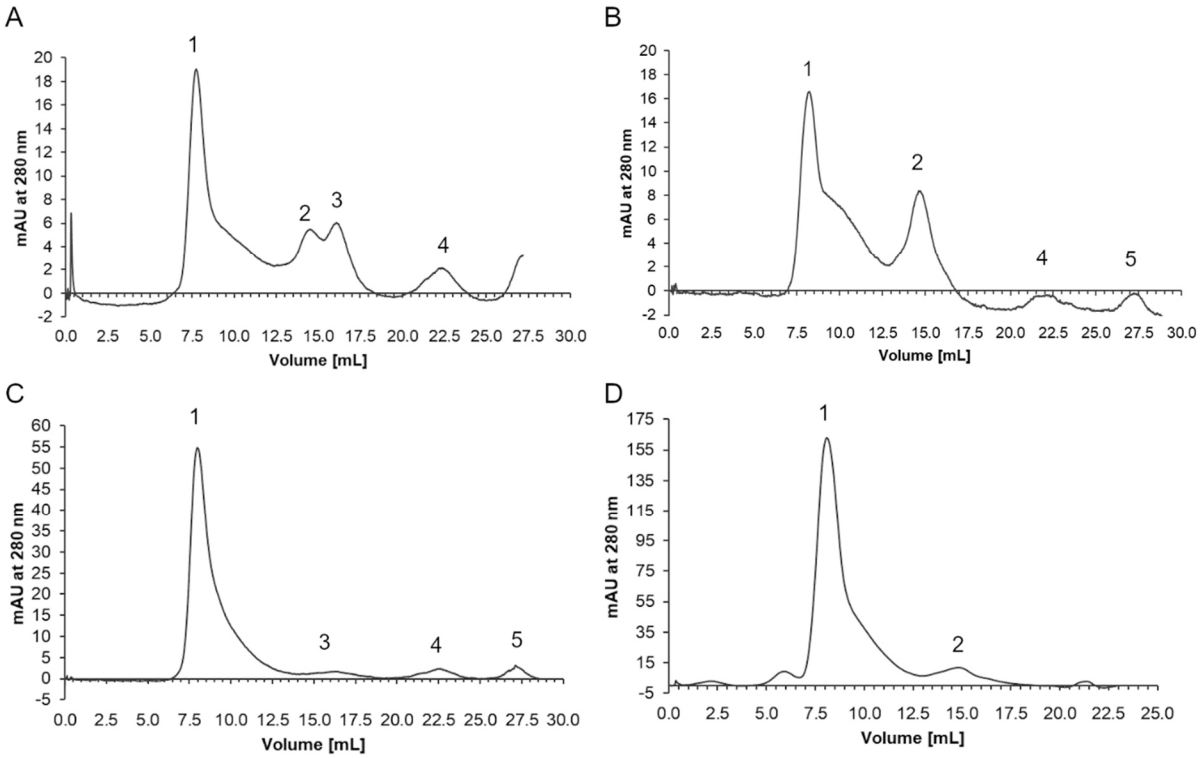

Figure 21: Absorption at 280 nm during analytical size exclusion chromatography. A: ATCC 25521, **B:** ATCC 27561, **C:** ATCC 14126, **D:** ATCC 7744.

After observing a lot of protein aggregation and also possible degradation, it was of great interest to find out if the storage conditions would have an impact in any way. For this reason, some fractions from the SEC were concentrated and stored in the fridge (4°C) overnight or shock frozen again, and then analysed for a second time. These chromatography runs led to rather high signal noise, as the collected fractions from the previous run had already contained very low amounts of protein. Still, some clear peaks could be detected (Figure 22).

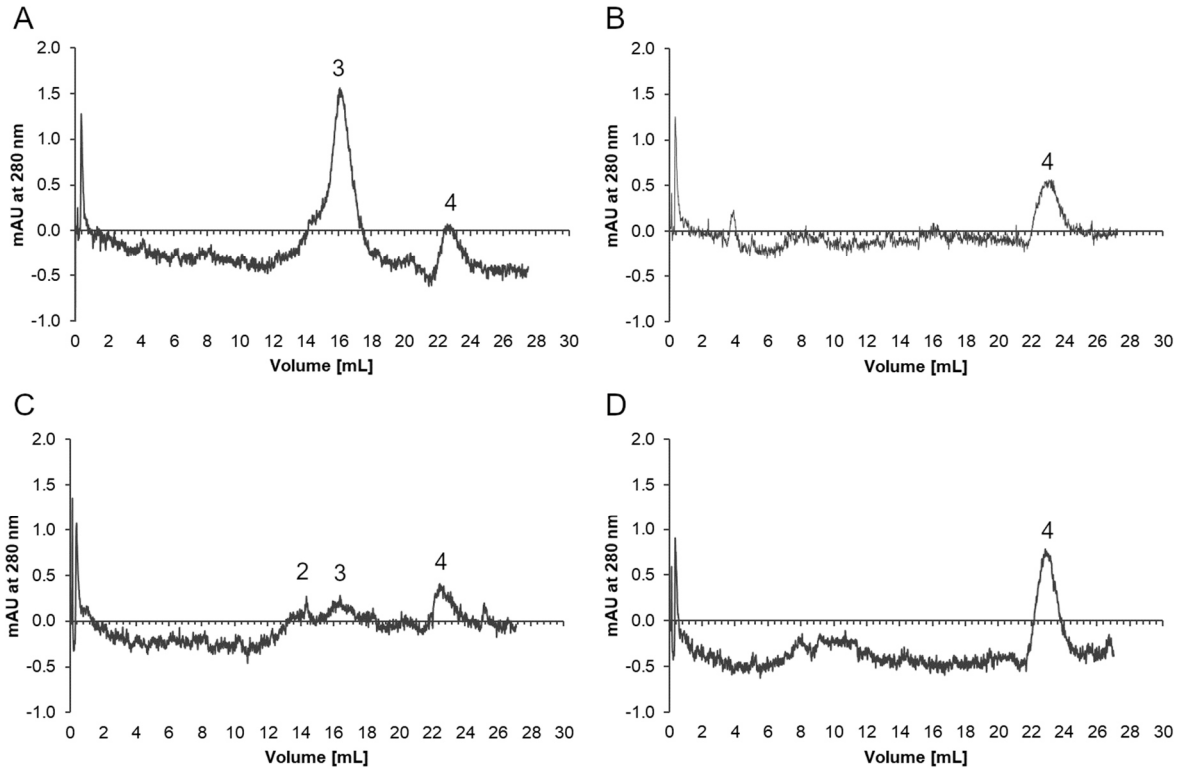

Figure 22: Size exclusion chromatography to analyse storage conditions. **A:** ATCC 25521 peak 3 (possible monomer) after storage at 4 °C overnight. **B** Peak 3 of panel A after shock freezing. **C:** ATCC 7744 peak 2 (possible dimer) after storage at 4 °C overnight. **D** ATCC 27561 aggregates after storage at 4 °C overnight.

Storing protein monomer samples at 4 °C overnight did not have a significant effect on the protein quaternary structure, still part of the protein seemed to have degraded (Figure 22, panel A). The protein monomers were collected and frozen in liquid nitrogen. After analysing this sample again via FPLC only small protein fragments were found (Figure 22, panel B). This led to the assumption that freezing single monomers could cause protein degradation. Anyway, at this point it was uncertain which subunit was purified here or if it was a mixture of both. When trying to examine the possible protein dimers the same way, only a very inconclusive chromatogram could be drawn (Figure 22, panel C). Keeping protein aggregates at 4 °C also appeared to result in protein degradation (Figure 22, panel D).

Although remaining protein quantities after SEC were rather low, selected samples were again analysed via SDS-PAGE. From the stained gel it was evident that the first peak in the chromatogram consisted of the wanted protein. It could be presumed that the aggregates were formed by both subunits, as two definite bands could be seen after denaturing with SDS. Moreover peaks 2 and 3 (dimer and monomer) apparently consisted of both subunits for the samples of ATCC 25521 and 27561. Peak 2 of ATCC 7744, which was thought to be protein dimer, actually showed a faint band at around 70 kDa on the gel (Figure 23). Some genes from the chaperone network, which were overexpressed in the TaKaRa strain, produce proteins of this

molecular weight. For example, DnaK (accession number AMH20900.1) has a molecular weight of 69 kDa and GroEL (ACT30875.1) 57 kDa. These impurities had already been detected previously on the SDS-gels after purification via Strep-Trap. So apparently, though the protein concentration of ATCC 7744 was the highest, the luciferase was primarily found in aggregates. However, the activity assay still resulted in 20 % bioluminescence intensity, whereas there was no activity found for ATCC 14126, which consisted mainly of aggregates as well.

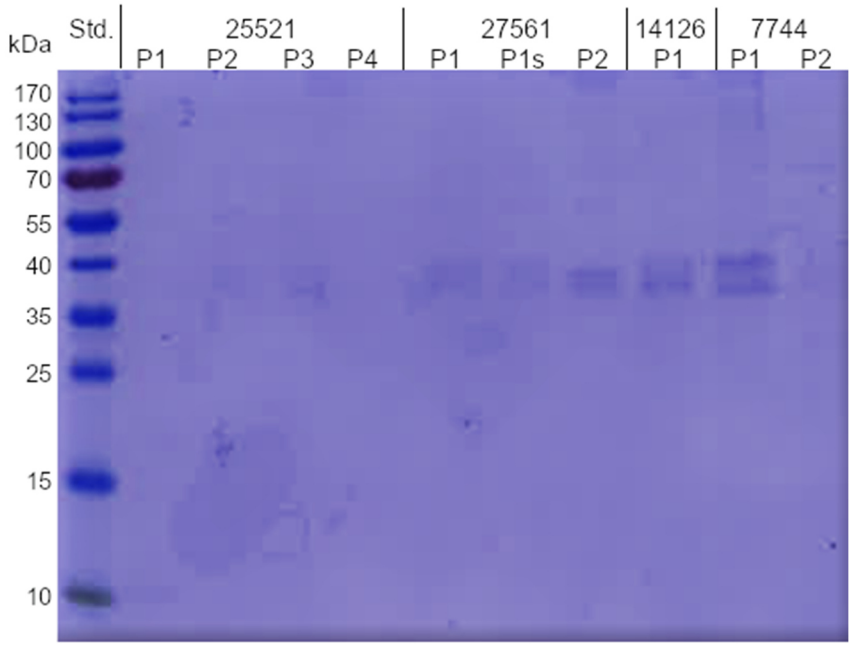

Figure 23: SDS-PAGE after SEC analysis. Selected samples were prepared for SDS-PAGE, though protein concentrations were low. The samples are labelled according to their corresponding peaks. P = peak, P1s = Peak 1 plus shoulder.

4.2 Light intensity in dependence of LuxF

Past studies had shown that LuxF might act as a scavenger of 6-(3′-(R)-myristyl)-FMN (myr-FMN) during the light reaction in bacteria, preventing the inhibition of the luciferase (59). To investigate if the presence of LuxF had a measurable effect on the intensity of bioluminescence, a previously developed approach was used, which was slightly adapted (64). The *lux* operons from *P. mandapamensis* ATCC 27561 and *P. leiognathi* ATCC 25521 were used. ATCC 27561 naturally contains *luxF* within this codon (*luxCDABFEG*) whereas ATCC 25521 does not. The created constructs allowed for simple deletion or addition of the *luxF* gene.

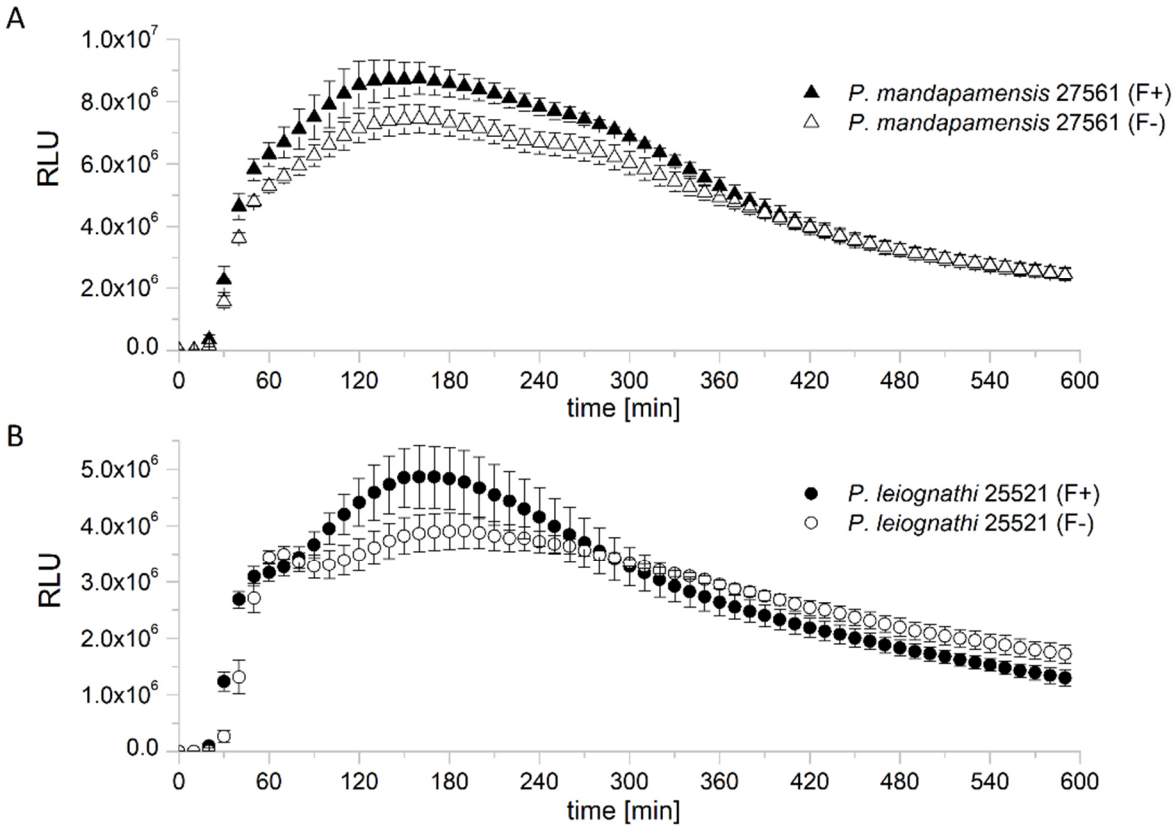

Figure 24: Relative light units (RLU) measured over time. *E. coli* systems with the *lux* operon of two different strains were compared, as well as the impact of LuxF on relative light emission. **A:** *E. coli* BL21 Star [pET28a] harbouring the *lux* operon of ATCC 27561, with and without *luxF*. **B:** *E. coli* BL21 Star [pET28a] harbouring the *lux* operon of ATCC 25521, with and without *luxF*.

The assay verified the above assumption that LuxF benefits bioluminescence intensity (Figure 24). For both systems, with natural *luxF* occurrence and where foreign *luxF* was introduced to the operon, the light intensity increased by a factor of 1.2. Still, bioluminescence levels between the two strains differed severely. *P. leiognathi* only produced 55 % of relative light units reached by *P. mandapamensis*, so introduction of *luxF* alone will not results in a massive rise in light production. The maximal relative light units were reached between 2.5 and 3 h after

induction with IPTG, strong differences in light intensity between strains with *luxF* and those without could already be seen after one to two hours.

As reference measurements, *E. coli* BL21 Star cells, *E. coli* BL21 Star [pET28a] cells and *E. coli* BL21 Star [pET28a] cells harbouring the *lux* operon with or without *luxF*, without induction with IPTG, were analysed (Figure 25). All cultures showed similar growth behaviour typical for bacterial cultures. Background bioluminescence could be detected in the non-induced samples, but at much lower levels compared to the experiments with induced strains. Light intensity only went up to a small fraction of the values acquired from the induced samples. Moreover, light emission did not start until 5-6 h after inoculation, whereas for induced cultures, a sharp increase in light intensity had already been detected after 30-60 min.

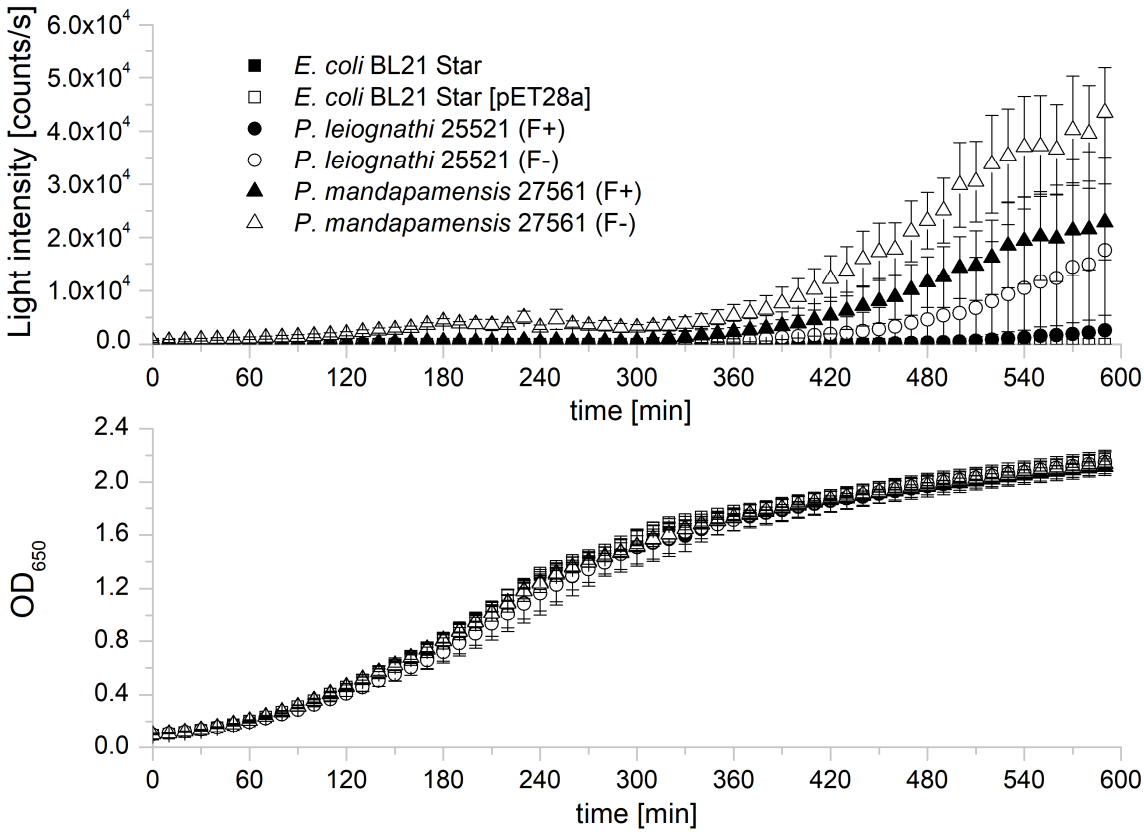

Figure 25: Reference measurements of light intensity and cell growth (OD_{650}).

5 Discussion

Bacterial luciferases have been studied for centuries. Still, there is yet a lot to learn regarding the mechanisms involved in the light reactions. It had been observed in the past that in the process of crystallization, only β_2-homodimer was obtained. The researchers suspected that the heterodimer collapsed at some point and the homodimer formed, omitting the α-subunit (48). To date, the only bacterial luciferase crystal structure available is from *Vibrio harveyi* ATCC 14126, with and without bound FMN (34, 42, 43). Therefore, obtaining pure active heterodimer is an important step towards studying complexed structures and protein-protein interactions.

The preparation of the vector constructs proved to be more challenging than anticipated. One of the issues was the absence of sequence information on six of the eleven luciferase genes to be cloned. To get around this limitation, primers were designed using the five available DNA sequences. These primer pairs were then used for related strains with the ambition that the primers would still bind to the specified regions of the gene if the sequences were similar enough. Moreover, A+T-content was quite high in the complementary regions, thus primers had to be designed rather long to reach adequate melting temperatures. Unfortunately, the primers only worked for the sequences they were designed for, leading to only five of initially eleven genes that could be amplified. These gene products were then digested and ligated into the vector to build constructs for expression.

To find a fitting expression host, three different *E. coli* strains were tested with all five constructs. Generally, the expression tests showed that the genes were overexpressed, though the bacterial luciferases behaved rather differently. Some luciferases seemed to be easier to express than others, showing no big difference between the used expression hosts. Others, in contrast, were only overexpressed clearly in one expression host. No universal expression host could be found, so two strategies had to be used. Three luciferases could best be produced in TaKaRa (ATCC 27561, 14126 and 7744) and one in *E. coli* BL21 Star (ATCC 25521). The fifth luciferase, TH1, was not studied further after the tests, as cell growth was slowed down substantially during expression.

To find the best purification strategy for all luciferases, tests with StrepTrap and HisTrap columns were performed. What was apparent from the SDS-gels as well as the Western Blots was that some protein residue was lost in the pellet. As this could be a consequence of insufficient sonication, the cell disruption method was improved by adding lysozyme prior to sonication for the large-scale protein production. During purification, small losses of protein due to insufficient binding to the column were observed with StrepTrap. Also, protein concentration in

the elution fractions was lower than after HisTrap column. From these differences in purified protein amounts, one could assume, that less α-subunit than β-subunit was produced, or some of it was degraded after biosynthesis. Since *luxA* was located upstream of *luxB* on the mRNA, as it is also found in nature, it was assumed that, if anything, *luxB* would be translated less. This leaves protein folding or stability issues to be the reason for the discrepancy in concentration. Strep-tagged low molecular weight products detected after large-scale purification were indicative of partial LuxA degradation. The Western Blots also showed that the purification of the β-subunit with HisTrap worked, but no α-subunit was detected in those samples. Whereas, when the α-subunit was purified with StrepTrap, β-subunit could be detected in the elution fractions, too. These results were also confirmed by MALDI-TOF analysis. This leads to the conclusion that dimer formation must have taken place. Knowing that β_2-homodimer is formed in the absence of α-subunit, it is possible that part of the LuxA produced was either misfolded or degraded, leading to an excess in LuxB. This would result in heterodimeric luciferase αβ as well as β_2-homodimer formation (40, 41).

Upon obtaining four recombinantly produced enzymes, which were purified with only few impurities, it was possible to compare and characterize them. An *in-vitro* activity assay was carried-out, in which activity was not measured by turnover but by light production. An already established assay was slightly adapted (19). The aldehyde serving as substrate, tetradecanal, was not fully soluble in water, therefore in this assay ethanol was used. The impact of this solvent change did not affect luciferase activity. Still, light emission peaked 1-2 s later than what the Brodl et al. paper reported. Levels of activity differed greatly between the investigated luciferases. Though *P. leiognathi* ATCC 25521 also reached lower relative light units in the *in-vivo* assays with the whole *lux* operon (55 %) compared to *P. mandapamensis* ATCC 27561, the gap to here is much bigger (4 %). Unfortunately, luciferase from *V. harveyi* ATCC 14126 was completely inactive. More information on the reasons for these differences was expected to be gained from size-exclusion chromatography.

It is known that non-dimeric protein still exhibits activity, but at far lower intensity (37, 38). Moreover, as discussed previously, formation of the stable homodimer β_2 could affect the amount of heterodimeric luciferase and therefore decrease light emission. Also, the protein concentration for the assay was not determined specifically for luciferase but spectrophotometrically at 280 nm. Discrepancies due to contaminations, protein degradation, monomer and aggregate formation could not be eliminated with this method. Therefore, more information on some of these factors was sought with analytical size exclusion chromatography. The chromatograms showed that all enzyme solutions were composed of mostly aggregates. However, the following SDS-gel showed that the aggregates were not formed by impurities but only α-

and β-subunits. These aggregates could have led to lower enzyme activity in general. Additionally, proteins with the molecular weight of 76 kDa and 35 kDa were found. These were suspected to be protein dimer and monomer. SDS-gel analysis proved that the 76 kDa peak was actually LuxAB in the ATCC 25521 and 27561 samples. Interestingly, ATCC 7744 showed the same peak, but with SDS-gel analysis it could be defined as protein impurity. So, in contrast to the prior assumption that aggregate formation might lead to loss of activity, luciferase of ATCC 7744 was active, even though LuxAB was present only in the form of aggregates. The inactivity of LuxAB from ATCC 14126 could be explained with SEC as well. No dimer peak was noticeable, and analysis of the aggregates indicated a shortage of α-subunit. Still, both subunits were present. This would suggest that no heterodimeric enzyme could be formed, instead other native states of the protein existed. From the Western Blots and the chromatography, it was established that some protein degradation must have occurred, presumably α-subunit for the most part. Low α-subunit concentration promotes the formation of β_2-homodimer, even though folding into the active heterodimer αβ is kinetically favoured (40, 41).

Obviously, protein degradation and especially aggregation was a big issue in this approach of heterodimeric enzyme production, so the influence of storage conditions was examined. And indeed, it was found that freezing protein monomers led to degradation, so did storing aggregates at 4 °C. The most interesting sample, the protein dimer, unfortunately could not be analysed, as concentrations were probably too low. Still, it can be assumed that the storage conditions chosen for the luciferases were not ideal. It should also be noted that the activity assay was carried out with frozen and then thawed protein, leading to unreliable data given the information that was obtained with SEC. Stability screens, e.g. thermal shift assays, would be helpful in finding more appropriate buffer conditions and suitable ways to preserve active protein. For example, protease activity could be inhibited by addition of EDTA to the storage buffer, as luciferase has already been reported to be prone to protease degradation. This degradation is limited to the mobile loop near the active centre on the α-subunit and leads to activity loss. Usually no further protein digest by proteases is observed (44, 45). Anyway, the Strep-tag detected in the immunoblot as part of the degradation product was at the N-terminus of LuxA, whereas the protease-labile loop is located near the C-terminal region. So, hydrolysis of peptide bonds of the mobile loop would not lead to the same results observed here, although it cannot be ruled out either.

The *in-vivo E. coli* assay with two different *lux* operons was used to find out if the protein LuxF had any influence on light intensity. It was shown that the presence of *luxF* in the operon led to a significant increase in light intensity, regardless of whether the gene was a natural component of the operon or not. This matches recent proposals that LuxF acts as a scavenger of

myrFMN and thereby aids the bioluminescence reaction. The effect was observed after 1-2 h, indicating that considerable levels of inhibition of the luciferase were not achieved before a significant amount of myrFMN was produced. Moreover, the operon taken from ATCC 27561 produced almost twice the light than that of ATCC 25521, regardless of the presence of LuxF. This indicates that other enzymes of these strains, apart from LuxF, differ in their efficiency in this experiment set-up. In the activity assay that was conducted with purified luciferases from these strains, the difference was even bigger. Anyway, due to reasons discussed earlier, the *in-vitro* assay cannot be used as reliable data for comparing luciferase activity at this stage. The minor light emission detected with samples, which carried the operon but were not induced, can be explained with the leakiness of the used T7/lac promoter system.

6 Conclusion

The aim of this thesis was to provide a foundation for future research on the structures of luciferases, including interaction studies with different molecules taking part in the process of light emission. The luciferases from strains *P. leiognathi* ATCC 25521, *P. leiognathi* TH1, *P. mandapamensis* ATCC 27561, *V. harveyi* ATCC 14126 and *A. fischeri* ATCC 7744 could be expressed and all except for TH1 were purified via the Strep-tag on the N-terminus of the α-subunit. The luciferases were then characterized and compared using different assays. An activity assay showed strong distinctions between the four purified luciferases, with ATCC 14126 even being entirely inactive. Size exclusion chromatography pointed to issues regarding enzyme stability as well as incorrect dimerization. Different oligomerization states of the protein were detected, which made a reasonable comparison of activity difficult, not knowing how much correctly folded protein was utilized. Investigation of the whole *lux* operon in *in-vivo* assays was more conclusive. The positive influence of LuxF on light emission could be shown for a strain naturally carrying *luxF* as well as for a strain where *luxF* was introduced into the operon. Furthermore, the light emission produced by the whole operon differed between ATCC 25521 and ATCC 27561, independent of the impact LuxF had.

With the information gathered, better ways to preserve the native quaternary structure of bacterial luciferase need to be established. To date, only the crystal structure of *V. harveyi* ATCC 14126's luciferase with and without FMN is available. Other bacterial luciferases as well as complexes with $FMNH_2$ and the aldehyde substrate would be vital to provide a deeper insight into the reaction mechanism behind bioluminescence.

7 List of Sources

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8 Appendix

Table 11: Primer sequences. The restriction sites are highlighted in blue.

Primer name	Sequence	T_m [°C]
TH1_pET51b_fwd	5'-CGGATCCGaaaattagtaatatttgtttttcataccaacc-3'	60
TH1_pET51b_rev	5'-GCGGCCGCtttaataaggttgtctttaatttttttgattaaac-3'	59
25521_pET51b_fwd	5'-CGTCGACaaaattagtaatatctgtttctcataccaa-3'	59
25521_pET51b_rev	5'-GCGGCCGCtttaataaggttatctttgatttttttgattaaac-3'	59
27561_pET51b_fwd	5'-CGGATCCGaaatttggcaatatttgtttctcata-3'	60
27561_pET51b_rev	5'-GCGGCCGCtagatttttcttgatttttttcattaaccatatta-3'	59
14126_pET51b_fwd	5'-CGGATCCGaaatttggaaacttccttctcactta-3'	62
14126_pET51b_rev	5'-GCGGCCGCcgagtggtatttgacgatgttg-3'	63
7744_pET51b_fwd	5'-CGGATCCGaagtttggaaatatttgtttttcgtatc-3'	61
7744_pET51b_rev	5'-GCGGCCGCgggtagattcttttcaatttttttgg-3'	62

```
;Script generated by script wizard (Omega V5.11 R3)
;Thomas Seidel, BMG LABTECH, January 2013 ... March 2014

;Protocol Names:
st1:="Eveline Biolumi_neu"
st2:="Eveline OD650_neu"
st3:="shake"

;Plate ID:
ID1:=""

;Number of readings (number of kinetic cycles):
NumberOfReadings:=60

R_Temp 28
wait for temp>=27.9

;================================================================================
=========

for Reading:=1 to NumberOfReadings do begin                ;kinetic loop
   ID2:="Wizard script 'LumiOD600_NEU', Prot.1"
   R_Run "<st1>"                                           ;execute first test protocol

   ;merge horizontal (kinetic):
   if Reading>1 then begin
      Call "MergeReadings.exe <DataPath> <User> H ID2"
   end;

   ID2:="Wizard script 'LumiOD600_NEU', Prot.2"
   R_Run "<st2>"                                           ;execute second test protocol

   ;merge horizontal (kinetic):
   if Reading>1 then begin
      Call "MergeReadings.exe <DataPath> <User> H ID2"
   end;

   ID2:="Wizard script 'LumiOD600_NEU', Prot.3"
   R_Run "<st3>"                                           ;execute third test protocol

end;                                                       ;end of kinetic loop

;end of script
```

Figure 26: Script for light intensity and OD_{650} measurements with ClarioStar Plus.

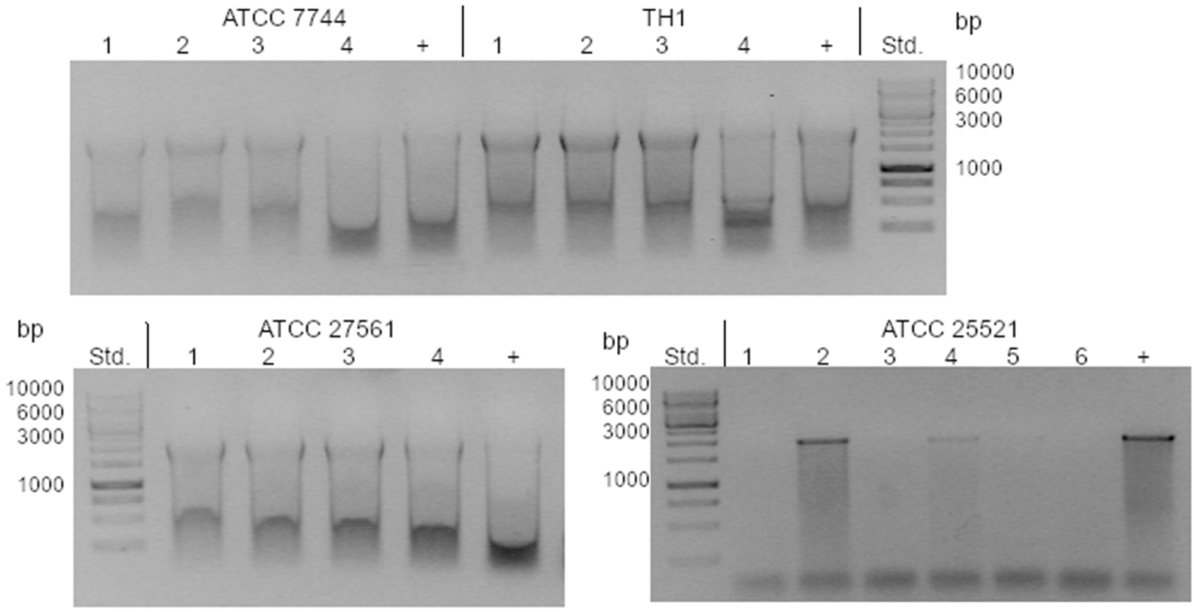

Figure 27: Agarose gels from colony PCRs of the remaining strains. + = positive control, Std. = 1 kb DNA ladder, bp = basepairs

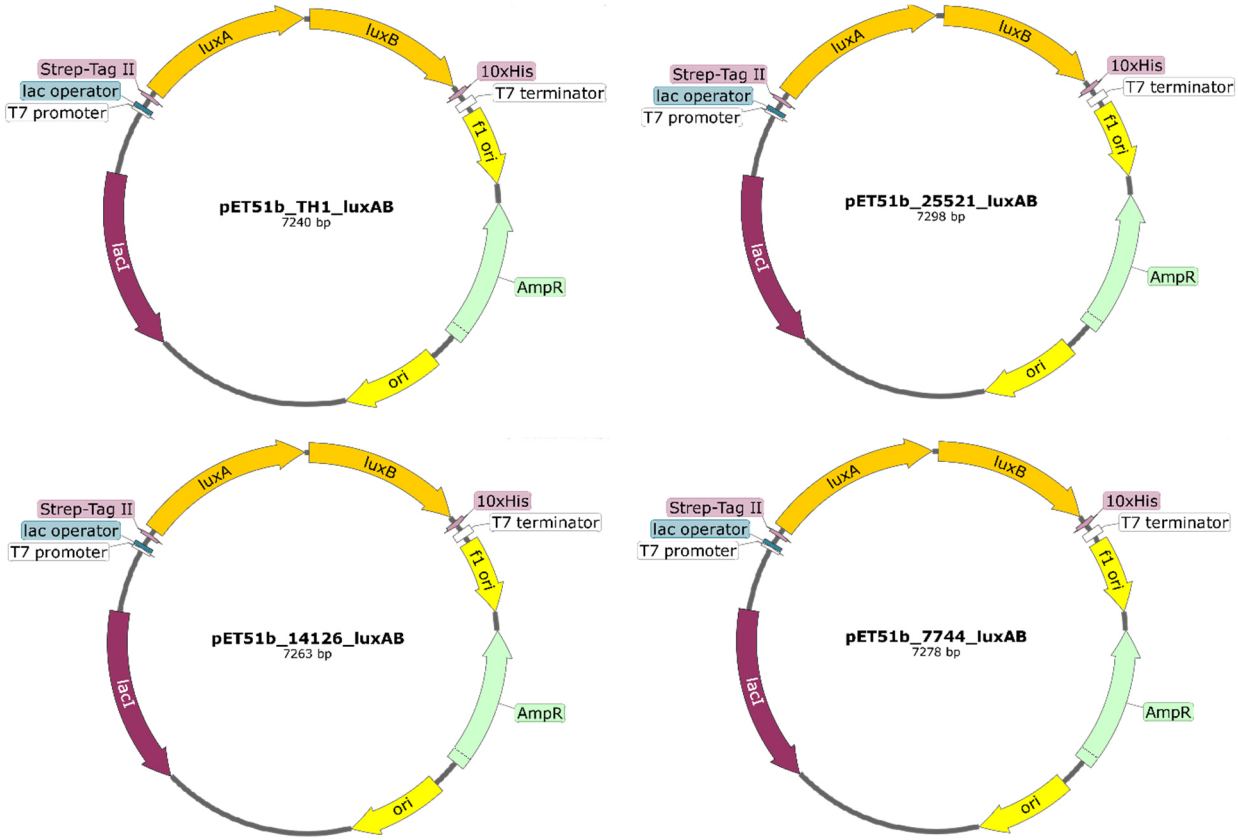

Figure 28: Additional plasmid maps of the constructs generated.