Evidence for the generation of myristylated FMN by bacterial luciferase

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Summary
The genes responsible for the light production in bioluminescent bacteria are present as an operon, luxCDABEG. Many strains of Photobacteria carry an additional gene, termed luxF. X-ray crystallographic analysis of LuxF revealed the presence of four molecules of a flavin derivative, i.e. 6-(3'-(R)-myristyl) flavin adenine mononucleotide (myrFMN) non-covalently bound to the homodimer. In the present study, we exploited the binding of myrFMN to recombinant apo-LuxF to explore the occurrence of myrFMN in various bioluminescent bacteria. MyrFMN was detected in all bacterial strains tested including Vibrio and Aliivibrio indicating that it is more widely occurring in bioluminescent bacteria than previously assumed. We also show that apo-LuxF captures myrFMN and thereby relieves the inhibitory effect on luciferase activity. Thus our results provide support for the hypothesis that LuxF acts as a scavenger of myrFMN in bioluminescent bacteria. However, the source of myrFMN remained obscure. To address this issue, we established a cofactor regeneration enzyme-catalyzed cascade reaction that supports luciferase activity in vitro for up to 3 days. This approach enabled us to unambiguously demonstrate that myrFMN is generated in the bacterial bioluminescent reaction. Based on this finding we postulate a reaction mechanism for myrFMN generation that is based on the luciferase reaction.

Introduction
Riboflavin, also known as vitamin B₂, serves as a precursor for the synthesis of flavin adenine dinucleotide (FAD) and flavin adenine mononucleotide (FMN), which share the same structural backbone, the isoalloxazine ring (Macheroux et al., 2011). Flavoenzymes use either FMN or FAD to carry out a plethora of redox reactions that mostly revolve around the activation of dioxygen and the handling of one-electron or two-electron transfer reactions (Massey, 1994; Joosten and van Berkel, 2007; Teufel et al., 2016). A prominent example for the utilization of dioxygen is the bacterial luciferase, which catalyzes the oxidation of long-chain fatty aldehydes to their corresponding fatty acids, e.g. tetradecanal to myristic acid (Scheme 1).

In the course of the reaction, reduced FMN reacts with dioxygen to the FMN-C4a-peroxide, which subsequently reacts with the aldehyde to the FMN-4a-hydroxide in an excited state, which relaxes to the ground state by emission of light centered at 490 nm. (Ulitzur and Hastings, 1979; Kurfurst et al., 1984). Detailed studies on the reaction mechanism led to the suggestion that a radical mechanism, such as the chemically-induced electron exchange luminescence (CIEEL) mechanism, is responsible for the population of the excited state (Eckstein et al., 1993).

The enzymes involved in bacterial bioluminescence are arranged in the form of an operon, with the typical gene organization being luxCDABEG as in Photobacterium leiognathi ATCC 25521 (Meighen, 1991). The luxAB genes encode for the heterodimeric protein luciferase consisting of a 40 kDa α-subunit and a 37 kDa β-subunit. The aldehyde required for the reaction is formed by a multi-enzyme complex consisting of a reductase (luxC), a transferase (luxD) and a synthetase (luxE). In addition, a NADH-dependent FMN reductase is encoded by luxG providing reduced FMN to the luciferase (Hastings et al., 1969; Boylan et al., 1985; Nijvapkul et al., 2008). In many photobacterial strains (like TH1, S1 and ATCC 27561) an additional gene ‘luxF’ was found in the operon inserted between luxB and luxE resulting in the new order, luxCDABFEG (Lee et al., 1991; Dunlap, 2009; Bergner et al., 2015). Interestingly, large quantities of LuxF were found in these bacteria. LuxF exists as a homodimer and shows an α/β barrel fold, similar to the β-subunit of the bacterial riboflavin synthetase.

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luciferase, and therefore has presumably arisen by gene duplication of luxB (Moore and James, 1994). The exact role of LuxF is not yet known, however it was hypothesized that the main function of LuxF is the binding of myristylated FMN (myrFMN), which is presumably a side product of the luciferase reaction. MyrFMN is thought to bind sufficiently tight in the active site of luciferase to inhibit the bioluminescent reaction (Moore and James, 1995). In fact, Wei and coworkers could demonstrate the inhibiting effect of myrFMN on the luciferase from Vibrio harveyi (Wei et al., 2001).

The generation of myrFMN in the marine bacterial strains is a largely unexplored phenomenon. In our previous report, we have reported a method to isolate myrFMN from P. leiognathi S1 (Bergner et al., 2015). Using isolated and purified myrFMN, we showed that it binds to recombinant apo-LuxF (K<sub>d</sub> = 80 nM) 50 times more tightly than to luciferase from P. leiognathi (K<sub>d</sub> = 4 µM) by using isothermal titration calorimetry. This tight binding to LuxF is clearly due to the large hydrophobic surface area of the myristyl group, which is deeply buried in each binding site. As a consequence, it is extremely difficult to remove the bound flavin derivative from LuxF and therefore harsh denaturing conditions are required to extract myrFMN from LuxF, as described previously (Bergner et al., 2015). Therefore, this tight binding of myrFMN to recombinant apo-LuxF was explored to exploit its occurrence in various bioluminescent bacterial strains (lux<sup>F<sub>3</sub></sup> and lux<sup>F<sub>7</sub></sup>) of Photobacteria. This analysis showed that myrFMN is present in all photobacterial strains tested, suggesting that its production is independent of the occurrence of luxF.

To better understand the occurrence and generation of myrFMN, we were interested in the relationship of bioluminescence intensity and the production of myrFMN in different bioluminescent bacterial genera, i.e. Aliivibrio, Vibrio and Photobacteria. These experiments suggested that total light production in bacteria correlates to myrFMN production and thus indicated that myrFMN is directly generated in the luciferase reaction. This insight prompted us to establish an enzyme driven cofactor regeneration system that sustains in vitro light emission for up to 3 days. We then utilized the apo-LuxF scavenging method to enrich and isolate any myrFMN produced in the experiment. This approach enabled us finally to unambiguously demonstrate that myrFMN is formed in the luciferase reaction.

### Results

#### Analysis of bacterial strains for light emission and myrFMN content

In a previous study, we have shown that 6-(3′-[R]-myristyl)-FMN (myrFMN) is generated by various bacteria in the genera Photobacterium and does not correlate with the presence of luxF, a gene encoding a protein that specifically binds myrFMN (Bergner et al., 2015). However, it remained unclear how myrFMN is generated and whether it affects the capability of bacteria to produce light. To address these issues, we have monitored the total light emission of various bioluminescent species and finally isolated myrFMN from the same bacterial cultures exploiting the high affinity of recombinant apo-LuxF as previously demonstrated (Bergner et al., 2015). The extraction protocol as described in experimental procedures was designed to ensure that protein-bound myrFMN is released and thus becomes available for re-binding to the added recombinant apo-LuxF. The scavenged myrFMN was then co-purified with the histidine-tagged LuxF by means of affinity chromatography and eventually released from the purified protein for quantification via HPLC. The results of the total light emission and myrFMN determination in seven different bioluminescent marine bacteria are summarized in Fig. 1. The highest total light emission and myrFMN content were found in P. leiognathi TH1 (this was set to 100% for further comparison) followed by strains S1 and 27561 respectively. However, only trace amounts of myrFMN were detected in strains 25521 and 25587. It was also tested if myrFMN production is confined to the genus Photobacteria or also occurs in other genera, i.e. Aliivibrio and Vibrio. As is evident from Fig. 1, the presence of myrFMN was demonstrated for A. fischeri and V. harveyi albeit in very small amounts in the case of the former species. These strains also produce less than 2% of light compared to the best light-emitting strain TH1. Thus, our results clearly indicate that generation of myrFMN occurs ubiquitously in bioluminescent bacteria and is not confined to Photobacteria. The presence of myrFMN extracted from the strains TH1 and S1 were further confirmed by HPLC-MS (Supporting Information). The amounts of myrFMN isolated from the other strains were below the detection limit for MS and
therefore were not analyzed in further detail. Furthermore, our findings suggest that myrFMN production and total light emission appear to be linked, suggesting that myrFMN is generated in the chemical processes leading to light emission, i.e. the oxidation of tetradecanal by bacterial luciferase. Interestingly, the best light emitters, i.e. _P. leiognathi_ TH1, S1 and 27561 possess the _luxF_ gene and thus produce LuxF, which may protect bacterial luciferase by scavenging myrFMN. In fact, we could recently demonstrate binding of myrFMN to photobacterial luciferase (Bergner _et al._, 2015), however, it remained to be shown whether binding of myrFMN affects the activity of the enzyme.

**Inhibition of luciferase by myrFMN**

Therefore, we set up an inhibition assay to evaluate the effect of myrFMN on the luciferase-catalyzed reaction. Briefly, purified myrFMN from _P. leiognathi_ 27561 was used in increasing concentrations (0–50 μM) in an assay with luciferase (200 nM) and other required components as mentioned in experimental procedures. The light emission over a period of 90 s was recorded. In Fig. 2A, it was shown that as the concentration of myrFMN increased, the intensity of light emitted decreased (reaction 1 – black filled squares). A decrease in light emission indicates inhibition of luciferase activity as the active site is obstructed by myrFMN and prevents binding of FMNH2. However, addition of recombinant apo-LuxF to the same reaction (after a primary cycle of 90 s) scavenged myrFMN and thus allowed binding of FMNH2 to be used in the bioluminescent reaction (reaction 1 + LuxF – red filled circles). In an additional reaction that more closely mimicked the situation _in vivo_, LuxF was added prior to initiating the reaction (reaction 2 – blue filled triangles). Increased light emission is observed at each concentration compared to the other two experiments suggesting that LuxF scavenged myrFMN before it bound to the luciferase in agreement with the 10-fold higher affinity to LuxF. This is also reflected by the IC50 for the inhibition as shown in Fig. 2B. The concentration of myrFMN required to reduce the light emission to 50% is approximately 2 μM, very close to the reported dissociation constant of myrFMN of 4 μM (Bergner _et al._, 2015). However, to inhibit 50% of the luciferase activity in the presence of LuxF, a six-fold higher concentration (~11 μM) of myrFMN is required, thus demonstrating the protective effect of LuxF on the bioluminescent reaction.

**In vitro multiple turnover reaction**

These findings point toward a direct link between light emission and myrFMN generation and thus we endeavored to provide direct proof that myrFMN is produced during the luciferase-catalyzed monoxygenation of long-chain fatty aldehydes. Toward this goal, we developed an _in vitro_ multiple turnover assay using the luciferase from _P. leiognathi_. This was achieved by coupling the bioluminescent reaction to YcnD, an oxidoreductase from *Bacillus subtilis* (Morokutti _et al._, 2005) and glucose dehydrogenase (GDH). YcnD reduces FMN at the expense of NADPH and thus provides FMNH2, which is stoichiometrically consumed in the luciferase-catalyzed reaction. On the other hand, GDH regenerates NADPH/ H+ from NADP+ by oxidizing D-glucose, which is added at a concentration to ensure prolonged luciferase activity leading to steady light emission. In this _in vitro_ multiple turnover reaction, light emission is linear for over 900 s, thus providing a suitable assay for studying the effect of myrFMN on the luciferase activity.
Detailed reaction conditions and reagent concentrations are described in experimental procedures. To rule out the possibility of artifacts, we performed the same experiment with several internal controls (viz. reaction with acid instead of aldehyde and without luciferase or oxygen) and none of the controls showed any myrFMN on the HPLC, therefore confirming that myrFMN is a product of the luciferase reaction and not a contaminant.

The reactions were started by the addition of luciferase resulting in intense light emission, clearly visible in the darkroom. The light intensity decreased over time and the reactions were stopped when light emission ceased (typically after ca. 72 h). After quenching the reaction, recombinant apo-LuxF was employed as before to isolate any myrFMN produced during multiple luciferase turnovers. Recombinant histidine-tagged LuxF incubated with the reaction mixture was then extracted by affinity chromatography and bound ligands were released by acid treatment and analyzed by a semi-preparative HPLC. As shown in Fig. 3, the main compound released from LuxF has a retention time identical to an authentic myrFMN sample isolated from *P. leiognathi* S1 (tR = 18.8 min). Moreover, the UV-Vis absorption spectrum is identical to the reference with absorption maxima at 386 and 441 nm (Fig. 3, insert).

(Scheme 2). The inhibition assays. A. Demonstrates inhibition of luciferase activity with increasing concentration of myrFMN. It is seen that as myrFMN concentration increases, the light emission decreases (reaction 1 – filled square). However, addition of LuxF to the same reaction (after primary cycle of 90 s) scavenges myrFMN allowing FMNH2 to follow the normal reaction and produce light (reaction 1 + LuxF – filled circles). A third condition, mimicking the *in vivo* situation, shows the inhibition effect of myrFMN in the presence of LuxF from the beginning of the reaction (reaction 2 – filled triangle). The peak maximum was plotted at each time point measured. B. clearly shows the concentration of myrFMN at which luciferase still emits 50% of light (IC50), which is approximately 1.7 μM. However, to inhibit 50% of the luciferase activity in the presence of LuxF, 6 fold higher concentration of myrFMN is required (≈11 μM), thus demonstrating the scavenging behavior of LuxF.

**Scheme 2.** Schematic representation of the multiple turnover *in vitro* assay. The luciferase employs molecular oxygen (O2) and reduced FMN (FMNH2) to oxidize, in this case, tetradecanal to tetradecanoic acid. For the reduced FMN a recycling system was established using the NADPH-dependent oxidoreductase YcnD from *Bacillus subtilis*. To recycle NADPH, glucose dehydrogenase (GDH) was applied.
MyrFMN samples were combined and dried under reduced pressure. The yield of myrFMN was approximately 0.18 nmol, thus only 0.005% of the FMN employed in our experiments was converted to myrFMN. The isolated and purified myrFMN was then subjected to HPLC-MS analysis using an Agilent Poroshell 120 SB-C18 column. Due to the usage of that column, the retention time of the myrFMN peak shifted to 14.2 min (Fig. 4A). Phosphorylated and dephosphorylated myrFMN have exact molecular masses of 681.29 and 601.31 respectively, and therefore a negative ESI scan mode from 100 to 800 m/z was used. The extracted ion chromatogram (XIC) at m/z 681 showed a distinct peak at the same retention time as the chromatogram at 370 nm, verifying the result (Fig. 4B). The mass spectrum confirms the formation of myrFMN (Fig. 4B, insert) showing a distinct peak at m/z 681.3. Thus the retention time observed in the HPLC system, the UV-Vis absorption properties and the determined mass of the isolated compound clearly show that myrFMN is generated in a luciferase multiple turnover reaction system.

Discussion

In our previous studies, we focused on the binding of myrFMN to LuxF and luciferase from P. leiognathi S1 (Bergner et al., 2015). We have shown that myrFMN binds to LuxF and luciferase with a $K_d$ of 80 nM and 4 $\mu$M respectively. Using this strong affinity toward LuxF, we showed that myrFMN is present in all photobacterial strains tested, irrespective of the presence or absence of luxF. In an extension of this work, we have demonstrated here that myrFMN is also produced in *Vibrio* and *Aliivibrio*. Furthermore, a quantitative analysis of light emission and myrFMN production showed a positive correlation suggesting that myrFMN is indeed generated as a consequence of luciferase activity (Fig. 1). Interestingly, the best emitting strains, i.e. TH1, S1 and ATCC 27561, were those featuring luxF prompting the question whether LuxF is prerequisite to high and sustained luciferase activity in vivo. To address this question, we have conducted a series of in vitro luciferase assays to probe the potential of LuxF to relieve the inhibitory effect of myrFMN on luciferase activity. As shown in Fig. 2, the presence of LuxF, tested in different experimental set-ups, unequivocally rescues luciferase activity in the presence of myrFMN and shifts the inhibition constant of myrFMN, as expressed by the IC$_{50}$, to higher concentrations. The IC$_{50}$ of $\sim$2 $\mu$M, as deduced in Fig. 2, is similar to the $K_d$ of myrFMN to luciferase reported earlier, i.e. $K_d = 4$ $\mu$M (Bergner et al., 2015), which in turn is similar to the binding constant of FMNH$_2$ to luciferase, i.e. $K_d = 0.8$ $\mu$M (Meighen and Hastings, 1971). Therefore, our results are in accordance with the relative affinities of myrFMN to LuxF and luciferase respectively (Bergner et al., 2015). The fact that preincubation with LuxF, mimicking the in vivo situation, leads to a substantially higher luciferase activity also suggests that dissociation of myrFMN from luciferase is a slow process compared to the binding to LuxF.

The obtained results clearly indicate a direct link between the formation of myrFMN and the luciferase catalyzed reaction. To provide a direct proof for this hypothesis, we designed an in vitro multi-enzyme, cofactor...
recycling system that sustained the luciferase reaction for at least 48–72 h. This allowed us to isolate sufficient material for chromatographic, spectroscopic and mass spectrometric analysis and clearly provided evidence that myrFMN is produced in the \textit{in vitro} luciferase reaction. This is the first direct experimental proof that myrFMN is formed in the luciferase catalyzed reaction.

\textbf{Proposed mechanism for myrFMN formation}

The demonstration that myrFMN is produced in the luciferase reaction invites the question how myrFMN generation can be rationalized based on the mechanism for the light-emitting process. It was shown that bacterial luciferase forms a stable FMN-4a-hydroperoxide intermediate, which subsequently reacts with the aldehyde substrate to form a FMN-4a-peroxyhemiacetal (Eberhard and Hastings, 1972; Kurfurst et al., 1984; Macheroux et al., 1993). The decomposition of this intermediate is the most critical step because it eventually leads to the population of the excited state of the FMN-4a-hydroxide intermediate (Scheme 3). The currently preferred models to explain the population of an excited-state FMN-4a-hydroxide are based on a radical mechanism, such as the chemically initiated electron exchange luminescence (CIEEL) process (Eckstein et al., 1993; Tu, 2013). In this mechanism, the decomposition of the FMN-4a-peroxyhemiacetal is initiated by the transfer of an electron from the N5 position of the flavin to the distal oxygen atom of the peroxylhemiacetal (Scheme 3). This triggers the cleavage of the O–O bond and the generation of an alkoxy radical. At this stage the proton from the C-1 carbon is abstracted and the resulting anionic radical transfers an electron back to the FMN-4a-hydroxide cation radical (Scheme 3, top line). This process is accompanied by the population of the excited state of the FMN-4a-hydroxide, which acts as the light-emitting luciferin.

Based on this radical mechanism we propose that the alkoxy radical rearranges to the carbon radical, as shown in Scheme 3, which then recombines with the FMN-4a-hydroxide radical cation to form a covalent bond between the C-3 carbon of the aldehyde and the C-6 carbon of the isoalloxazine ring. Rearomatization and cleavage of water will then lead to the formation of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{HPLC-MS measurement of the isolated myrFMN sample of the \textit{in vitro} assay.}
\end{figure}
the flavin adduct. It should be noted that this leads to the formation of the myristylaldehyde linked to the flavin rather than myristic acid. Because aldehydes are prone to oxidation, we assume that this may occur spontaneously after formation of the flavin adduct. It is important to emphasize that this model rationalizes how a rather unreactive saturated carbon atom is activated to form a covalent carbon–carbon bond. Because no other mechanism put forward for the luciferase catalyzed reaction has the potential to explain the formation of myrFMN, its very occurrence supports a radical mechanism for the luciferase reaction.

Given the very low yield of myrFMN obtained after up to 3 days of turnover in vitro we assume that the bioluminescent reaction efficiently outruns the formation of the side product. Nevertheless, our data show that bacterial strains that are capable of producing LuxF produce significantly more light apparently because LuxF scavenges myrFMN and thereby prevents the inhibition of the luciferase. Therefore, the creation of luxF, presumably by gene duplication of luxB, was an important evolutionary invention that provided an enormous advantage over other bioluminescent bacteria.

Experimental procedures

Chemicals

Tetradecanal, IPTG, FMN, NADPH, glucose and all buffer components were from Chemos GmbH, Sigma-Aldrich, Peqlab and Roth. All these chemicals were used directly without further purifications. GDH was a gift from Dr. Wolfgang Kroutil, University of Graz.

Instrumentation

UV-Vis absorption spectra were recorded using a Specord 205/210 spectrophotometer (Analytic Jena, Jena, Germany). Both, in vivo (bacterial bioluminescence) and in vitro light emission (single and multiple turnover reactions), were measured using a Centro LB960 microplate luminometer (Berthold Technologies, Bad Wildbach, Germany). All protein purifications were primarily performed using a 5 ml HisTrap FF affinity column (GE Healthcare, UK) and later applied to a HiLoad 16/600 Superdex 200 prep grade gel filtration column on an AKTAexplorer 100 Pharmacia Biotech (GE Healthcare life sciences, UK).

HPLC analysis was performed with a semi-preparative Dionex UltiMate 3000 equipped with a Dionex UltiMate diode array detector. Separation over an Atlantis dC18
(4.6 × 250 mm, 5 μm) column (Waters) was achieved using a gradient of 0.1% TFA in water and acetonitrile at 25°C and 1 ml min⁻¹ flow rate starting from 0% acetonitrile to 95% within 20 min, holding 95% for 5 min and going down to 0% within 5 min and holding 0% for another 10 min. For determination of the peaks the wavelengths at 280, 370 and 450 nm were used respectively. For evaluation of the results, up to four references were measured having the following retention times and wavelength maxima: FAD: tₚ = 9.1 min; maxima: 372/447 nm; FMN: tₚ = 9.8 min; maxima: 371/446 nm; riboflavin: tₚ = 10.4 min; maxima: 370/445 nm; myrFMN: tₚ = 18.7 min; maxima: 386/442 nm. Fractions were collected from 18.0 to 24.0 min and fraction tubes were changed every 15 s. Those fractions containing myrFMN, according to the analysis at 370 nm, were combined and dried under reduced pressure. This sample was dissolved in methanol for HPLC-MS analysis.

HPLC-MS analysis was performed on an Agilent Technologies 1200 Series system equipped with a MWD SL multiple wavelength detector (deuterium lamp, 190–400 nm) and with a single quadrupole LCMS detector using electrospray ionization source (ESI). The samples were separated over an Agilent Poroshell 120 SB-C18 (3 × 100 mm, 2.7 μm) column using the same gradient as mentioned above but with 0.01% FA in water and acetonitrile at 25°C and 0.5 ml min⁻¹ flow rate. Determination of the peaks was achieved by analyzing the chromatograms at 210, 280, 370 and 450 nm respectively and by using the mass spectrometer.

A negative ESI-Scan mode from m/z 100 to 800 was used to evaluate all the peaks and corresponding masses. The application ‘XIC’ of the HPLC-MS software ChemStation was utilized for linking the peaks with the m/z values 601 (dephosphorylated 6'-[(3'-R)-myristyl] FMN; C₃₁H₄₅N₄O₅; exact mass 601.31)) and 681 (phosphorylated 6'-[(3'-R)-myristyl] FMN; C₃₁H₄₇N₅O₁₁P; exact mass 681.29)). By using this function one defined m/z value (in this case 601 or 681) is searched within the negative ESI-scan mode from m/z 100 to 800 and drawn as separate chromatogram by the program.

**Bioluminescent bacterial strains**

The following bacterial strains were selected for our study: TH1, S1, ATCC 27561, ATCC 25521 and ATCC 25587 from genus *P. leiognathi*; ATCC 14126 from genus *V. harveyi* and ATCC 7744 from genus *A. fischeri*. Only TH1, S1 and ATCC 27561 contained luxF as reported previously (Bergner et al., 2015). The bacteria were cultivated in 1 l flasks with 200 ml of 246-SWC media (246-Sea water culture). The cells were grown at 25°C with 130 r.p.m. shaking for optimal aeration.

**Cloning, gene expression and production of proteins**

Production and purification of LuxAB and LuxF from *P. leiognathi* ATCC 27561 and of YcnD from *B. subtilis* was performed as described previously (Morokutti et al., 2005; Bergner et al., 2015). The protein concentrations were calculated using the molar extinction coefficients 82,335 M⁻¹ cm⁻¹ (*P. leiognathi* LuxAB) and 26,025 M⁻¹ cm⁻¹ (*LuxF*) at 280 nm and 12,190 M⁻¹ cm⁻¹ (*YcnD*) at 450 nm respectively.

Based on the DNA sequence available, a synthetic gene for LuxAB (from *V. harveyi* ATCC 14126 and *A. fischeri* ATCC 7744) was designed with a C-terminal octa-histidine tag and optimized for *Escherichia coli* codon usage. The gene was then sub-cloned into a pET24b vector (KanR) and transformed into *E. coli* Rosetta strain (CmpR) for heterologous expression. The cells containing the construct were grown in LB media with kanamycin (50 μg ml⁻¹) and chloramphenicol (20 μg ml⁻¹) at 37°C to an OD₆₀₀ of ~0.6. The protein production was then induced by addition of 0.1 mM IPTG and the cells were further grown for 16 h at 20°C. The cells were then harvested by centrifugation (7,000 g, 10 min, at 4°C) and the wet cell pellets were stored at −20°C until further use. The proteins were purified similar to the photobacterial luciferase as reported previously (Bergner et al., 2015). The protein concentrations were calculated using the molar extinction coefficients 84,230 M⁻¹ cm⁻¹ (*V. harveyi* LuxAB) and 83,200 M⁻¹ cm⁻¹ (*A. fischeri* LuxAB) at 280 nm.

**Analysis of bacterial strains for light emission vs. myrFMN content**

Assays were performed using the 96 well white assay plates. Light emission was measured using the luminometer. The OD of the cells was obtained by absorption measurements using an UV-Vis spectrophotometer at 660 nm (instead of 600 nm) to exclude the interference of artefacts due to bioluminescence.

To measure the light emission, 100 μl of cells were pipetted into each well of the assay plate and after an initial delay of 1 s and rapid mixing of the plate for 0.1 s, the reading was taken for 1 s total time. The readings were taken for 24 h with time intervals 30/60 min. The light intensity (counts) at each time point was plotted. For comparison, the following bioluminescent bacterial strains were taken into consideration: *P. leiognathi* TH1, S1, ATCC 27561, ATCC 25587 and ATCC 25521; *V. harveyi* ATCC 14126; *A. fischeri* ATCC 7744.

For the quantification of myrFMN content, 10 g of wet cell pellet was taken for each strain mentioned above. The extraction of myrFMN in vivo, using recombinant apo-LuxF was performed precisely as described previously (Bergner et al., 2015). The isolated product was analyzed with HPLC.

**Inhibition assay**

In this assay, the light emitted by the enzyme luciferase was measured using the luminometer. The reaction was performed similar to the *in vitro* assay described previously (Brodl et al., 2017). The assay was performed in 96 well black assay plates using 100 mM potassium phosphate buffer with 300 mM NaCl, pH 7 as reaction buffer.

Briefly, the reaction mixture contained 200 nM luciferase, 300 nM YcnD, 300 nM FMN, 500 nM NADPH and the substrate-buffer suspension in the reaction buffer to make...
up the final volume to 250 μl (due to the low solubility of aldehyde in the buffer, concentrated aldehyde suspensions were obtained by mixing 5 μl of aldehyde with 10 ml of buffer using ultra-sonication). Apo-LuxF was used in excess concentrations (up to 100 μM) as a scavenger for myrFMN. The reactions were started by injecting NADPH to the master mix (after a delay of 5 s) and the readings were subsequently taken every second for 90 s total time.

Pure myrFMN sample in a concentration gradient from 0 to 50 μM was used as the inhibitor in this assay (extracted and purified as reported previously in Bergner et al., 2015). The concentration of myrFMN was calculated using the extinction coefficient of 11,350 M⁻¹ cm⁻¹ at 396 nm. Three different sub-assay conditions were used to observe the inhibitory effect on the luciferase activity: First, in the absence of LuxF; second, in the presence of LuxF from the beginning of the reaction; and lastly when LuxF is added at a later stage of the reaction (after 90 s). All three conditions were tested for increasing myrFMN concentrations. Light emission was plotted against myrFMN concentration. The control reactions without myrFMN in each condition were considered as 100%.

In vitro multiple turnover reaction

All enzymes and cofactors were diluted and prepared in 100 mM potassium phosphate buffer pH 7. For multiple turnover reactions the following components and concentrations were used: 100 μM LuxAB, 150 μM FMN, 150 μM YcnD, 2 mM NADPH, 200 mg GDH (lyophilized powder), 1 M glucose and 100 μl substrate-buffer solution. Due to low solubility of aldehydes in water, a substrate-buffer solution was obtained by adding 40 μl of tetradecal to a mixture of 1,900 μl reaction buffer and 100 μl Triton X. This led to complete solubility of the substrate. All components were mixed to a final volume of 25 ml in a 50 ml enzyme reactor. The reaction was started by the addition of LuxAB and stirred at 4°C for a maximum of 72 h. After six hours, 200 μM BSA was added to stabilize the enzymes.

For the workup, three main steps were performed analogous to the extraction of myrFMN in vivo (Bergner et al., 2015). The reactions were stopped by adding 2.5 g guanidine-HCl and dropwise concentrated HCl to lower the pH to ~2. Three consecutive extractions were made with 15 ml each of an organic mixture of ethyl acetate:butanol (1:1). The organic phase was separated by centrifugation (4,566 g at 4°C for 30 min) and the collected, unified organic layers were dried in a vacuum evaporator at 56°C under reduced pressure. The residual powder was dissolved in 20 ml lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8) and incubated with excess of recombinant histidine-tagged apo-LuxF for 30 min in the dark. The apo-LuxF with bound myrFMN was loaded on a 1 ml HisTrap FF/HP column (GE Healthcare) for purification. The column was washed with wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8) and the fractions were eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 300 mM imidazole, pH 8). The eluant fractions were pooled and concentrated to 500 μl. As a final purification step organic extraction was repeated again in small scale as described above. The dried samples were analyzed by HPLC.

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Author contributions

CRT and EB expressed and purified all proteins and cofactors. CRT designed the experiments, performed the assays and analyzed the data for the myrFMN vs. light correlation in bacterial strains and determined the IC₅₀ for the inhibition assays. CRT and EB designed and performed the in vitro multiple turnover assays. EB analyzed the results for in vitro assays via HPLC. EB further confirmed the cofactor molecule on HPLC-MS. CRT, EB and PM wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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