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Bright stable luminescent yeast using bacterial luciferase as a sensor

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Abstract

Bright luminescent yeast cells with light intensities similar to bacteria containing luciferase (LuxAB) were generated by providing saturating nontoxic levels of the substrates for the bioluminescence reaction ($FMNH_2 + O_2$ and fatty aldehyde $\rightarrow$ light). Z-9-Tetradecenal added to yeast (+*lux*AB) gave a luminescent signal close to that with decanal with the signal remaining strong for >24 h while luminescence of yeast with decanal decayed to less than 0.01% of that with Z-9-tetradecenal after 2 min. Moreover, yeast survived in 0.5% (v/v) Z-9-tetradecenal while 0.005% (v/v) decanal was lethal. Luminescence of yeast (+*lux*AB) was also stimulated 100-fold by transformation with the NADPH-specific FMN reductase (FRP) from *Vibrio harveyi*. The recognition of the nontoxicity and high luminescence generated by Z-9-tetradecenal and the generation of high levels of $FMNH_2$ in yeast by transformation with a flavin reductase provide evidence for the strong potential use of bacterial luciferase as the light-emitting sensor of choice in eukaryotic organisms.

Keywords: Aldehyde; Bacterial luciferase; Bioluminescence; Yeast; FMN reductase; Z-9-tetradecenal

The bacterial *lux* system from luminescent bacteria can be very effectively expressed in different bacteria including many pathogens. This system acts as a light-emitting sensor that can be used to monitor not only gene expression but also the survival and distribution of the bacterial species [1–5]. Light emission is generated simply by transfer of the five *lux* genes, *lux*CDABE, under a suitable promoter into the nonluminescent bacteria as there is an ample internal supply of the substrates, $FMNH_2$ and O_2, for the reaction catalyzed by luciferase (LuxAB). The third substrate, a long chain aldehyde, can be produced from the fatty acyl-ACP (-CoA) in the cell by LuxCDE using endogenous ATP and NADPH [6]. Alternatively, only the *lux*AB genes need to be introduced into the bacteria as the aldehyde [4], usually decanal, can be added and will pass readily through the cell wall to generate light.

Application of the bacterial *lux* system to generate light-emitting eukaryotes including yeast has met with far less success primarily because of difficulties in supplying the flavin and aldehyde substrates in vivo necessary for the luminescence reaction. Earlier studies have shown that a fused bacterial luciferase from *Vibrio harveyi* (*lux*A-B) can be effectively expressed in yeast but luminescence was relatively low on addition of decanal [7,8]. The low level of luminescence arose as $FMNH_2$ is not sufficiently high in the cytoplasm of eukaryotes to saturate the bacterial luciferase; much higher levels are believed to be present in the mitochondria. Moreover, decanal, the aldehyde used in all bioassays with bacterial luciferase, has been found to be lethal to yeast, leading to the conclusion that the bacterial *lux*AB reporter genes cannot be used in these model eukaryotic organisms [9] at least for continuous monitoring of the same cells.

In this report we show that Z-9-tetradecenal gives high levels of light in yeast containing bacterial luciferase (LuxA-B) without causing toxicity and that complementation of yeast (+*lux*A-B) by *V. harveyi frp*, a gene coding for a NADPH-dependent FMN reductase [10], generates light intensities in yeast at least l00 times brighter than those previously measured using the bacterial *lux* genes [7,8]. Moreover, the yeast remained viable on addition of exogenous Z-9-teradecenal allowing

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continuous monitoring of luminescence in the same sample during growth over extended periods of time.

Experimental procedures

Materials and strains. Z-9-Tetradecenal was purchased from Bedoukian Research and decanal was purchased from Sigma. Yeast (*Saccharomyces cerevisiae*) was strain *W303-1A*, *MATa*, *leu2-3*, *112*, *ura3-1*, *his3-11 15*, *trp1-1*, and *ade2-1*. Restriction enzymes, T4 DNA ligase, and Vent DNA polymerase were from New England. Biolabs Research Laboratories. PCR primers were synthesized by Invitrogen.

Plasmids. Fused *V. harveyi* bacterial luciferase (*lux*A-B) was produced as described previously by a simple mutation of *lux*A eliminating the stop codon and fusing it in-frame by a 10 amino acids linker with *lux*B [7]. The initiation Met codon of *lux*B was also changed from ATG to CAG (Gln) and an *Eco*RV site was introduced directly preceding the *lux*A ATG initiation codon. The fused *lux*A-B was transferred as an *Eco*RV–*Bgl*II fragment into a BamHI site after the *GAL1* promoter in an *Escherichia coli*/yeast vector pLG5 derivative (ura^+, amp^r) to generate pLG-luxA-B.

The gene coding for *frp* was cloned by PCR of *V. harveyi* chromosomal DNA using primers synthesized by Invitrogen. A *Not*I site was inserted in the forward primer nine nucleotides prior to the ATG initiation codon of *frp* while a *Xho*I site was inserted in the reverse primer 94 nucleotides after the TAA stop codon. The PCR product was restricted by *Not*I and *Xho*I, purified on 1% agarose, stained with ethidium bromide, and then excised and purified using the Geneclean II kit (Q-Biogene). The purified DNA was initially ligated into the *Not*I and *Xho*I sites in the single copy vector pGREG505 (amp^R, leu^+) between the *GALl* promoter and the *CYC1* terminator. The pGR-frp was then isolated by selecting for ampicillin resistant transformants in *E. coli*. The *frp* gene with the *CYC1* terminator was then cloned using primers extending from immediately after the upstream *Not*I site to immediately before a *Kpn*I site just after the sequence coding for the *CYC1* terminator. A *Spe*I and a *Pst*I site were included at the ends of the forward and reverse primers, respectively. The PCR product was digested with *Spe*I and *Pst*I and purified as described above before ligation into the same sites in the multicopy pRS425 plasmid (amp^R, leu^+) just after the *GALL* promoter [11]. The pRS-frp was isolated by selecting for ampicillin resistant transformants in *E. coli.*

Transformation and growth of yeast. Yeast (*S. cerevisiae*) cells were transformed with the different plasmids after treatment with lithium acetate [12]. Transformed cells were selected at 30 °C on 1.5% agar plates containing 0.67% yeast nitrogen base medium supplemented with 2% galactose (Sigma) and a 0.1% SC dropout media omitting uracil and/or leucine for selection of transformants. Yeast were grown in liquid culture in the same media minus agar in a rotary shaker at 30 °C and the growth was followed by measuring the absorbance at 600 nm (OD_{600}).

Luminescence assays. In vivo luminescence was generally determined from the maximum light emission in light units (LU) for 1 ml of culture after mixing with 1 μl of aldehyde by vortexing for 15 s. One light unit corresponds to 4×10^9 quanta/s based on the light standard of Hastings and Weber [13].

Results

A nontoxic aldehyde for generation of bacterial luciferase activity in yeast

As decanal is lethal to yeast [7], we tested unsaturated long chain aldehydes that are relatively volatile and soluble and give high luminescence with bacterial luciferase [14] to determine if they can cross the yeast membrane and have low toxicity. Addition of Z-9-tetradecenal to yeast containing the fused bacterial luciferase gene (pLG-luxA-B) gave a maximum luminescence response very similar to that with decanal. However, even more significant was the survival of yeast at all added concentrations of Z-9-tetradecenal. Fig. 1A shows that yeast grown with different concentrations of aldehydes for 24 h in liquid culture and then plated on solid media survived at concentrations of Z-9-tetradecenal (Z_9) as high as 0.5% (v/v) while yeast mixed with decanal (C_{10}) were killed at concentrations as low as 0.005% (v/v). Exposure of the surviving yeast colonies to Z-9-tetradecenal or decanal vapor resulted in the emission of light (Fig. 1B) but only the cells exposed to Z-9-tetradecenal vapor and not decanal vapor continued to emit light if monitored between 2 and 4 h after initial exposure (Fig. 1C). Light could even be detected in yeast (pLG-luxA-B) after five days upon exposure to fresh aldehyde provided the yeast cells had only been previously exposed to Z-9-tetradecenal. However, once exposed to decanal vapor, the cells remained dark (data not shown) even on addition of fresh Z-9-tetradecenal or decanal vapor.

Stimulation of luminescence in yeast by FRP flavin reductase

Although light emission can be detected in yeast containing the *lux*AB genes on adding exogenous aldehyde, the level of light intensity is far less than that

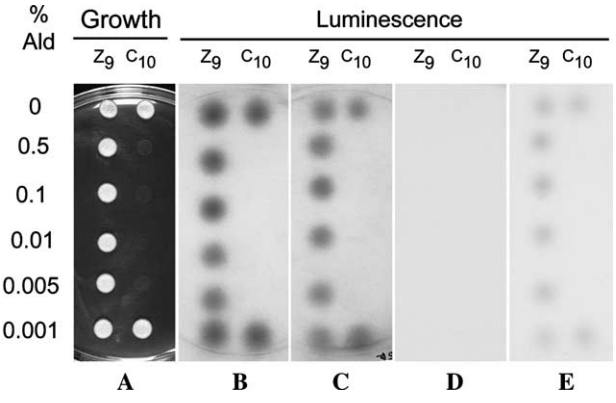

Fig. 1. Growth and luminescence response of yeast colonies (pLG-luxA-B) exposed to decanal (C_{10}) and Z-9-tetradecenal (Z_9) after growth for 24 h with 0.001–0.5% (v/v) of aldehydes in liquid medium. The same volume of cells was then plated on solid media and grown for an additional 24 h (A). Exposure to C_{10} (B) or Z_9 (C) vapor for 2 h gave light only for colonies grown with Z_9 or $\leqslant$0.001% C_{10} (B). Light is no longer being emitted from yeast after 2 h exposure to C_{10} vapor (D) but is still being emitted from cells exposed previously to Z_9 vapor (E). Light emission in panels (D) and (E) was recorded for 2 h after the same cells were exposed for 2 h to aldehyde in panels (B) and (C), respectively.

observed in native or recombinant luminescent bacteria. Extraction of high levels of functional luciferase from yeast [7] indicates that the low light intensity in vivo arises from insufficient $FMNH_2$ in the cytoplasm to saturate the active site of luciferase. As $FMNH_2$ is chemically oxidized very rapidly by oxygen, it is likely not to be produced at a high enough rate in the cytoplasm due to the levels of FMN, NADP (H), and/or NAD (P)H-dependent FMN reductase being too low.

Complementation of yeast (pLG-luxA-B) with a plasmid containing the *V. harveyi frp* gene coding for NADPH–FMN reductase [10] resulted in a large stimulation of light compared to yeast (pLG-luxA-B) in the absence of *frp*. Fig. 2 shows that over the entire growth range, yeast (pLG-luxA-B, pRS-frp) cells are about 100 times brighter than yeast (pLG-luxA-B) cells upon addition of Z-9-tetradecenal to 1.0 ml aliquots at any stage of growth. A similar result was obtained upon addition of decanal instead of Z-9-tetradecenal. The luminescence intensity per unit of cell growth ($LU/ml/OD_{600}$) was constant over the entire growth range having an intensity of 30 $LU/ml/OD_{600}$ corresponding to 1.2×10^{11} $quanta/s/OD_{600}$ close to that observed for luminescent recombinant or native bacteria containing *V. harveyi* luciferase ($3–10 \times 10^{11}$ $quanta/s/OD_{600}$; note OD_{600} of cells $\cong 1.3$ OD_{660} of cells). Expression of the fused *lux*A-B gene under the strong pT7 promoter by T7 RNA polymerase in *E. coli* gave a light intensity of 2.4×10^{11} $quanta/s/OD_{600}$ with decanal [7] only twice as high as that observed for the luminescent yeast with Z-9-tetradecenal in the present experiments.

Continuous monitoring of yeast using bacterial bioluminescence

Expression of the bacterial *lux*AB genes in yeast using decanal as the aldehyde substrate precludes repeated analyses of the same sample due to the toxicity of the decanal substrate. However, as Z-9-tetradecenal is not toxic, it should now be possible and more convenient to repeatedly analyze the same sample for luminescence expression.

Fig. 3 shows a plot of luminescence (LU/ml) on mixing yeast (pLG-luxA-B, pRS-frp) with decanal or Z-9-tetradecenal over 5 min. Light intensity for yeast (pLG-luxA-B, pRS-frp) initially is slightly higher with decanal than with Z-9-tetradecenal. However, the light intensity rapidly decays with decanal while the light with Z-9-tetradecenal drops by about 70% over the first two min and then remains constant over the next 5 min. Indeed by 2 min after mixing, luminescence intensity with Z-9-tetradecenal is over 10^4 times higher than with decanal. After about 3 min absolutely no light can be detected with our instrumentation for yeast cells (+*lux*-AB, +*frp*) mixed with decanal while light intensity with

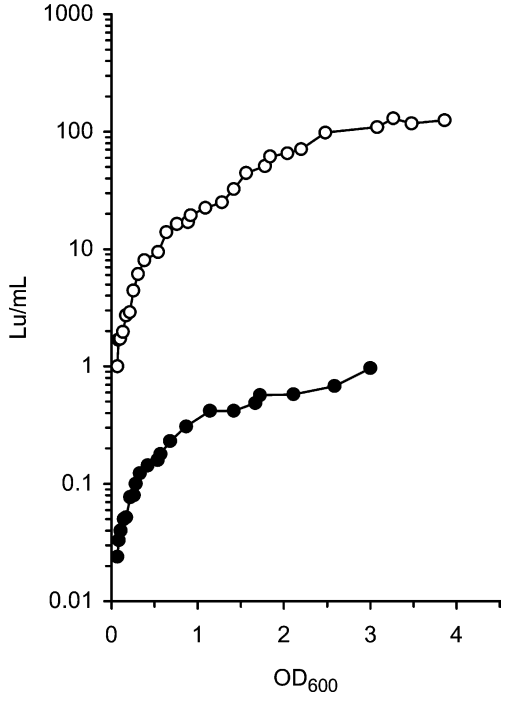

Fig. 2. Dependence of luminescence during growth of yeast (pLG-luxA-B) on the presence of the *frp* gene coding for NADPH-dependent FMN reductase (*frp*). Cells were grown in the complete liquid media minus uracil and leucine. Yeast (pLG-luxA-B, pRS) (●); yeast (pLG-luxA-B, pRS-frp) (○). Luminescence in LU/ml was measured using Z-9-tetradecenal.

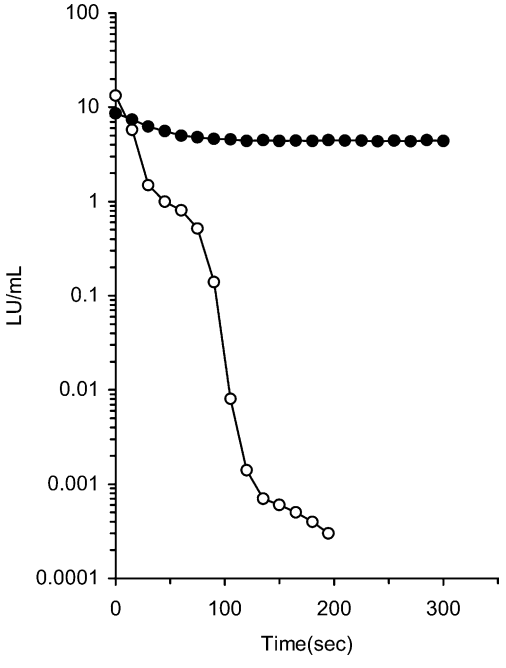

Fig. 3. Dependence of luminescence on time of incubation with aldehyde. One ml of yeast cells (pLG-luxA-B, pRS-frp) at an $OD_{600} = 0.9$ was incubated with either 1 μl of Z-9-tetradecenal (●) or 1 μl of decanal (○). All points were measured after vortexing 5 s. Luminescence with decanal was below the level of detection after 200 s.

Z-9-tetradecenal remains high and continues to do so for hours and even days.

To determine the effects of aldehyde on the luminescence and growth on yeast (+*lux*A-B, +*frp*) over a longer period of time, yeast cells (+*lux*A-B, +*frp*) were inoculated at different cell densities in the presence and absence of aldehyde and the extent of growth and luminescence was determined 24 h later. Table 1 shows that after 24 h, yeast (+*lux*A-B, +*frp*) grew in the presence of 0.1% Z-9-tetradecenal at a rate that was similar to but somewhat slower than that in the absence of aldehyde. This difference in growth rate for yeast (±0.1% Z-9-tetradecenal) is dependent on the initial inoculum with Z-9 having little effect on the growth rate at higher inocula. As expected, yeast cells did not grow in the presence of decanal and luminescence could not be detected even after the addition of Z-9-tetradecenal. In contrast, after 24 h of incubation of the yeast cells with 0.1% (v/v) Z-9-tetradecenal, the luminescence intensity has risen greater than 10-fold. Addition of fresh Z-9-tetradecenal to the cells results in a further increase in light giving levels of luminescence more comparable to that observed for yeast cells grown for 24 h without aldehyde. It should be noted that the light intensity on adding Z-9-tetradecenal to yeast cells that have never seen aldehyde tends to decrease with time upon mixing by vortexing over the first few minutes (see Fig. 3) while light intensity in cells previously mixed with Z-9-tetradecenal tends to rise if vortexed over a few minutes (data not shown) presumably due to the release of previously bound aldehyde. As all assays were uniformly conducted after only 15 s of mixing, the differences between the luminescence of samples (± Z-9-tetradecenal) are greatly amplified compared to analyses after 1–2 min particularly for cultures of high cell density after long periods of incubation.

To ascertain if luminescence can be used to monitor the effects of reagents that block the growth of yeast, tunicamycin, cycloheximide, and fluconazole were incubated with yeast (+*lux*A-B, +*frp*) and Z-9-tetradecenal (Table 1). After 24 h, the growth of yeast was inhibited by all three compounds as measured by the OD_{600} of the cell culture. Comparison of the light intensity to the control cells containing Z-9-tetradecenal alone shows that the light intensity was decreased by 10–40-fold (or 20–80-fold on adding fresh Z-9-tetradecenal) by these inhibitors, indicating that luminescence can provide a strong index of the toxic effects of these compounds on yeast.

Table 1
Effect of aldehyde[a] and inhibitors on luminescence and growth of yeast

Conditions	OD_{600}	LU[b]	LU (+Z_9)[c]
No additions, $t = 0$	0.1	0.001	2.4 ± 0.1
No additions, $t = 24$ h	4.2	0.020	63.5 ± 5.1
Z_9 for 24 h	3.7	5.4 ± 1.6	19.8 ± 5.6
Z_9, cycloheximide[d] for 24 h	0.11	0.14 ± 0.01	0.28 ± 0.06
Z_9, tunicamycin[d] for 24 h	0.19	0.52 ± 0.02	0.96 ± 0.14
Z_9, fluconazole[d] for 24 h	0.51	0.34 ± 0.05	0.94 ± 0.08
C_{10} for 24 h	0.1	0.000	0.000

[a] Aldehydes were added to the media at a concentration of 0.1% (v/v) just after inoculation with yeast (pLG-luxA-B, pRS-frp). Z_9, Z-9-tetradecenal; C_{10}, decanal.

[b] Light units (LU) were measured directly for 1 ml of cells.

[c] LU were measured just after the addition of fresh 0.1% (v/v) Z_9.

[d] Cycloheximide was added at 2 μg/ml, tunicamycin at 2 μg/ml, and fluconazole at 60 μg/ml.

Discussion

The results with yeast demonstrate that, Z-9-tetradecenal, a substrate for the luminescence reaction catalyzed by bacterial luciferase, can readily be added to yeast without causing toxicity even at concentrations of 0.5% (v/v). Moreover, luminescence stays at relatively high levels and decays very slowly removing the necessity to add fresh aldehyde substrate even after long periods of incubation. This behavior contrasts sharply with that of decanal, which is highly toxic to yeast; luminescence of yeast with decanal reaches a maximum in the first 20 s and then decays rapidly with a signal not being detected after 5 min. The reason(s) for the difference in toxicity of the two aldehydes is not known. It is likely that Z-9-tetradecenal binds to luciferase at a much lower concentration than decanal as the apparent affinity of luciferases for aliphatic aldehydes has been shown to increase with chain length [15]. A low solubility of Z-9-tetradecenal and/or rate of transport of this aldehyde into yeast would expose the cytoplasm to a lower effective concentration of the aldehyde, thus reducing the toxicity compared to decanal but still allowing saturation of the luciferase active site with aldehyde. The possibility that there may be differences in the metabolism of the two aldehydes by the yeast biochemical machinery could also explain the differences in toxicity.

In any case, the large difference in toxicity (>100-fold) to yeast between decanal and Z-9-tetradecenal was clearly surprising. This difference in toxicity and the strong luminescence response of yeast (+*lux*A-B) to Z-9-tetradecenal that continued over long periods of time (24 h or longer) permit the continued monitoring by light emission of the same samples of yeast. Not only does this permit more rapid and convenient analyses, but yeast can now be readily analyzed under conditions in which it is difficult to repeatedly remove samples for analyses including monitoring of the growth and distribution of yeast on other organisms. A major bonus in using Z-9-tetradecenal in bioassays is its relative low volatility compared to decanal making it much more readily amenable, having a lower odor and being safer for use in the laboratory. As Z-9-tetradecenal is commercially available, inexpensive, and easily manipulated

and delivered as a liquid, just as is decanal, it might be the aldehyde of choice for analyzing recombinant bacteria as well as yeast containing *lux*AB.

A potential alternate choice to adding aldehyde is to clone into yeast the *lux*CDE genes coding for the fatty acid reductase complex that converts fatty acyl precursors in the cell into fatty aldehyde (i.e., tetradecenal). This approach would remove the need to add any aldehyde provided that the fatty acyl precursors present in bacteria are also readily available in the yeast cytoplasm and the *lux*CDE genes can be readily expressed in eukaryotes. Experiments have now been initiated to clone the *lux*CDE genes under separate promoters into the same plasmid with the *lux*AB and *frp* genes and then transform yeast with the plasmid as well as to recombine the *lux*CDE, *lux*AB, and *frp* DNA into the yeast genome.

The large stimulation of the luminescence levels upon complementing yeast (pLG-luxA-B) cells with a gene coding for the *V. harveyi* NADPH-dependent FMN reductase provided strong evidence that the limiting parameter in generating $FMNH_2$ for the bacterial bioluminescence reaction in yeast is the level of FMN reductase and not the levels of NAD(P)H or FMN. The *V. harveyi* FRP NADPH-dependent FMN reductase was chosen as there is good evidence to suggest that the FRP protein interacts directly with *V. harveyi* luciferase and channels the $FMNH_2$ directly to the luciferase active site [16]. Whether this interaction played a key role in generating sufficient $FMNH_2$ for the reaction catalyzed by luciferase in the yeast cytoplasm is not known. It certainly would be of interest to determine if other FMN reductases can be utilized to stimulate luminescence in yeast (+*lux*) cells and if they are as effective as the *V. harveyi* FRP reductase. Alternatively, the use of luciferase genes from other bacterial species with *V. harveyi* FRP reductase could be considered as *V. harveyi* FRP is reported to interact with luciferases from other species.

In the present experiments, we were fortunate in complementing two multicopy vectors (pLGDS5 and pRS425) containing the fused luciferase (*lux*AB) and the flavin reductase (*frp*). Experiments complementing the multicopy vector with *lux*AB (pLG-luxA-B) with a single copy vector (pGREG505) containing *frp* (pGR-frp) in yeast resulted in the levels of luminescence intermediate between yeast (pLG-luxA-B) and yeast (pLG-luxA-B, pRS-frp). These results suggest that luminescent yeast strains with *lux*A-B and *frp* recombined as a single copy in the yeast genome will give reasonable levels of light, albeit dependent on the specific promoter [11]. Future experiments will focus on recombination of the genes into the genome as this approach is much more readily applied to species such as *Candida albicans* than transforming with plasmids. The use of inhibitors (cycloheximide, tunicamycin, and fluconazole) of fungal growth on the light emitting-yeast demonstrates that in vivo luminescence arising from bacterial luciferase can function as a direct and sensitive reporter of yeast survival providing an excellent screening mechanism for the detection of new antifungal compounds.

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