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Toxicity of the bacterial luciferase substrate, *n*-decyl aldehyde, to *Saccharomyces cerevisiae* and *Caenorhabditis elegans*

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Abstract This study determined that the bacterial luciferase fusion gene (*lux*AB) was not a suitable in vivo gene reporter in the model eukaryotic organisms *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. *Lux*AB expressing *S. cerevisiae* strains displayed distinctive rapid decays in luminescence upon addition of the bacterial luciferase substrate, *n*-decyl aldehyde, suggesting a toxic response. Growth studies and toxicity bioassays have subsequently confirmed, that the aldehyde substrate was toxic to both organisms at concentrations well tolerated by *Escherichia coli*. As the addition of aldehyde is an integral part of the bacterial luciferase activity assay, our results do not support the use of *lux* reporter genes for in vivo analyses in these model eukaryotic organisms.

Key words: Biosensor; Luciferase; Bioluminescence; Toxicity

1. Introduction

The *lux*-mediated light reaction relies on cellular energy ($FMNH_2$), therefore light output can be used to directly quantify the activity of cells in vivo. Such an insight is of great importance for any study involving the metabolism of cells, for example in cytotoxicity analyses. The *lux* genes required for self luminescence are often present in the host genome in the order *lux*C, *lux*D, *lux*A, *lux*B, *lux*E and are transcribed as a polycistronic mRNA in a bacterial host. As polycistronic mRNA is inefficiently processed in eukaryotes, the luciferase coding genes *lux*A and *lux*B have been fused to enhance expression. Several versions of fused luciferase proteins have been constructed [1–6]. For this study a *lux*AB fusion created by Boylan et al. [2], which has 80% activity of wild-type luciferase at 30°C, was studied for its suitability as a reporter gene in *Saccharomyces cerevisiae* and, potentially, *Caenorhabditis elegans*. The addition of a fatty acid aldehyde (commonly *n*-decyl aldehyde) substrate is required for luminescence to occur. Cytotoxicity assays based on the firefly luciferase light output have been developed for both *S. cerevisiae* and *C. elegans* and are described elsewhere [7,8]. Through application of these cytotoxicity assays it was determined that the aldehyde substrate was a potent toxin to eukaryotic cells at concentrations well tolerated by bacterial cells.

2. Materials and methods

2.1. S. cerevisiae, C. elegans and Escherichia coli strains and growth conditions

The *S. cerevisiae* strain used in this study is *W303-1B* (*MAT*α, *leu*2-3,112 *his*3-11, 15 *trp*1-1, *can*1-100, *ade*2-1, *ura*3-1) as described by Thomas and Rothstein [9]. *S. cerevisiae* were transformed with plasmids using the 'Quick and easy transformation procedure' [10]. *S. cerevisiae* cultures were grown at 30°C in Synthetic Complete (SC) medium. SC medium was prepared as described by Strathern [10]. For strain selection SC without uracil was used. The *C. elegans* strain used in this study was PE39 (*fels1*[*rol-6*(*su1006*)*let-858*::*luc*Δ]; described by Lagido et al. [8]). This strain carries *luc*Δ gene under the control of the *C. elegans* constitutive promoter *let-858* [11]. Standard conditions were used for *C. elegans* cultivation as described by Lewis and Fleming [12]. The nematodes were cultured in complete S medium [13] supplemented with 40 g l^{-1} of *E. coli* OP50 [14], at 25°C and 160 rpm in an orbital shaking incubator for 5 days. The *E. coli* strain pLUC, containing the pGL2 control vector from Promega, was used in this study as a control. It was cultured in L-broth (tryptone, 10 g; NaCl, 10 g; yeast extract, 5 g; distilled water, 1 l), supplemented with 100 μg ml^{-1} ampicillin, at 37°C with shaking.

2.2. Vector construction for luxAB expression in S. cerevisiae

The vector pGLUXP (Fig. 1) is based on the yeast centromeric plasmid pRS316*GAL*I [15]. A 300 bp fragment of a *PGK* terminator was amplified by polymerase chain reaction from YCpPLP [16] using primers designed to introduce restriction enzyme sites for placing the terminator at the 3′-end of the polylinker. The S5R primer (5′-GTGTTGCTTTCTTATCCGCGGAGAAATAAATTGAAT-3′) introduced a *Sac*II site at the 5′-end of the terminator region and a *Sac*I site was inserted by the S3R oligonucleotide (5′-TTTTTCGAA-ACGCAGAGCTCTCGAGTTATTAAACTT-3′) at the 3′-end. During *lux*AB (2.1 kb) amplification a *Xba*I site was introduced by the primer X5R (5′-CGTAATACCAACAAATCTAGAAATGTTATG-AAATTT-3′) to the 5′-end of the gene and a *Not*I site was introduced by the primer N3R (5′-AAGGGGCATCGCGGCCGCTTCAGCAT-CAGTTAAACG-3′) at the 3′-end.

2.3. Bacterial luciferase activity analyses for S. cerevisiae

For luciferase activity analysis, 1 ml of medium was removed and 5 μl of *n*-decyl aldehyde was added to the culture. Luminescence was quantified in a BioOrbit 1251 luminometer using a *Multiuse* software package (version 1.01/April 1991/JN). The luminescence was monitored over a 6 s period and a mean value calculated by the software. The units of luminescence were amended to RLU (relative light units) which equated to 10 mV s^{-1} ml^{-1}. All assays were performed at 25°C.

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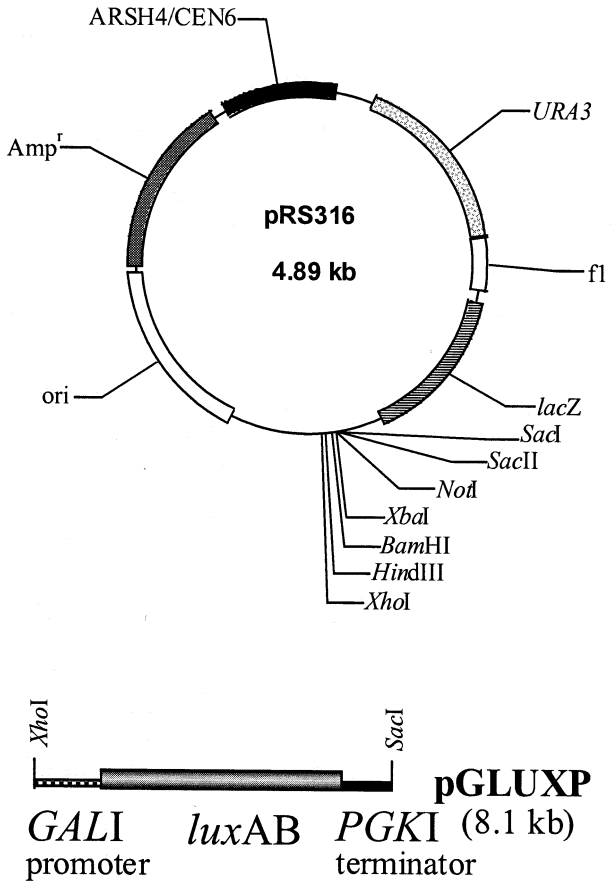

Fig. 1. Vector created for episomal expression of luciferase in *S. cerevisiae*. The luciferase expression cassette was placed into the polylinker of pRS316 using the restriction enzyme sites indicated. The luciferase gene inserted into this vector is a fusion of the *lux*A and *lux*B genes from *Vibrio harveyi*, created by Boylan et al. [2].

2.4. Effects of n-decyl aldehyde on actively dividing S. cerevisiae

S. cerevisiae (containing pGLUXP) cultures were grown at 30°C in an orbital shaking incubator at 200 rpm. At an OD_{600} of around 0.3, half of the culture volume was placed into sterile flasks (preincubated at 30°C) and allowed to grow until OD_{600} was around 0.5. At this stage, 200 μl of *n*-decyl aldehyde (equivalent to 5 μl per ml) was added to half of the total number of flasks. The effect of this addition on the growth of *S. cerevisiae* was then monitored through hourly measurements of optical density.

2.5. Comparison of S. cerevisiae, C. elegans and E. coli exposure to n-decyl aldehyde

The yeast lucΔ strain was prepared for bioassay analysis as described in Hollis et al. [7]. To each well, 85 μl of ddH_2O was added and then 10 μl of cells, washed and prepared in 0.1 M KCl, was added. A range of aldehyde dilutions were then added (in 5 μl volumes) to the respective wells. The aldehyde was diluted in 100% ethanol and 5% ethanol was added to the wells containing 0% aldehyde in order to determine maximum bioluminescence. Cells were incubated for 5 min and 100 μl of citrate phosphate buffer (pH 2.5) containing 0.2 mM luciferin was added to the wells. Luminescence was quantified in a Lucy Anthos I (Anthos Instruments, Austria) 96-well microplate luminometer using the *Stingray* (v2.0b31) software package. The Lucy Anthos I luminometer monitored luminescence over a 1 s period and a mean RLU value was calculated by the *Stingray* software package. These bioassays were performed in triplicate at 25°C. *C. elegans* were prepared as in Lagido et al. [8]. Nematodes were then incubated for 30 min with 0.1 mM luciferin, at 25°C and 160 rpm, then split into four replicate 1 ml samples, with 1.8×10^4 nematodes per ml. *E. coli* pLUC was grown to an OD of 1.1, washed and resuspended in citric phosphate buffer at pH 5.5. Luciferin was added to a concentration of 0.1 mM. Light output was measured by a BioOrbit 1251 luminometer in RLU. The signal was integrated over 12 s (nematodes) or 6 s (bacteria), at 25°C with shaking. Light output from worms or bacterial samples was measured 3 min before exposure to *n*-decyl aldehyde and their mean 100% luminescence value determined. The samples were exposed to the various concentrations of *n*-decyl aldehyde by adding 1 μl of pure or diluted aldehyde (in ethanol) and luminescence was measured after 3 min. Pure ethanol (1 μl) was added to controls.

3. Results and discussion

Luminescence of the yeast pGLUXP strain was 50 to 100 times that of background, indicating that a functional *lux*AB fusion protein was being produced. Luminescence was not increased through the addition of more than 5 μl of *n*-decyl aldehyde substrate per ml of culture. Others studies, including Hill et al. [4] and Almashanu et al. [1], stated that the limited pools of $FMNH_2$ could be a major inhibitory factor to high levels of luminescence in *S. cerevisiae* cells. Another possible inhibitory factor, thermostability, was not found to be a major influence as experiments performed at 25 and 30°C did not indicate any significant differences in light output (data not shown). Interestingly, the light output kinetics of the pGLUXP strain was that of a flash rather than a long sustained glow. A second peak of luminescence could not be induced through the addition of further substrate or increased oxygen saturation. Even, if the concentration of aldehyde was decreased then only the initial peak of luminescence was lowered, without preventing the decay in luminescence. As additional substrate did not induce further luminescence, these data suggested possible toxic effects of the aldehyde substrate.

Results represented graphically in Fig. 2 clearly demonstrate the adverse effect from the addition of the aldehyde substrate on growth of *S. cerevisiae*. No growth was observed on plates spread with exposed cells, even after several days. This indicated that exposure to *n*-decyl aldehyde was not just preventing growth, but resulted in yeast cell mortality. Additional cytotoxicity assay results indicate that an EC_{50} (effective concentration resulting in a 50% decrease in maximum bioluminescence) value for *n*-decyl aldehyde is likely to be less than 0.05% for yeast (Fig. 3). The concentration used in assays for *lux*AB derived luminescence quantification was 10-fold greater than the EC_{50} value indicated from this assay.

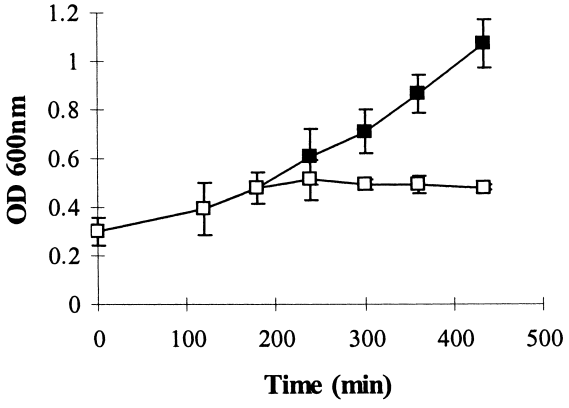

Fig. 2. *S. cerevisiae* growth following addition of *n*-decyl aldehyde after 180 min. The addition of aldehyde (□) results in growth arrest. The growth of the control culture (■) increased with time, as expected. This experiment was carried out in triplicate at 30°C and the error bars represent standard error of the mean triplicate value.

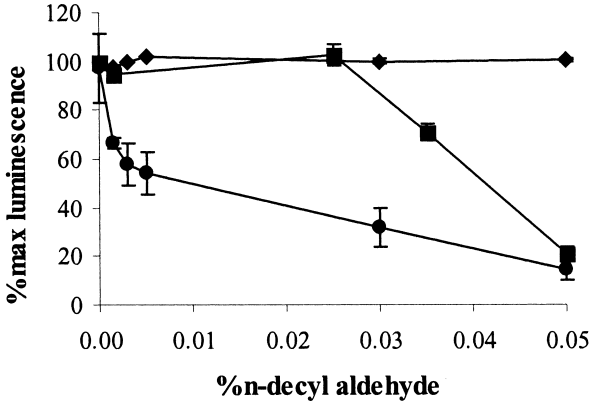

Fig. 3. Effect of *n*-decyl aldehyde on luminescent eukaryotic biosensors and bacterial cells. The toxic effects of *n*-decyl aldehyde could be detected by the *S. cerevisiae* cells (■) after only a 5 min exposure in ddH_2O. An estimated EC_{50} value of less than 0.05% was observed. The effects of 3 min exposure of nematodes (●) or bacteria (◆) to *n*-decyl aldehyde are shown. An estimated EC_{50} value of less than 0.03% was observed for nematodes. The *n*-decyl aldehyde was added to the bioassay dissolved in ethanol. The maximum concentration of 0.1% ethanol was included in the control measurements. Light output is shown as a percentage of the maximum luminescence, representing the mean value of three or four samples and error bars show standard error.

To ascertain if this response was yeast specific, the effect of *n*-decyl aldehyde on *C. elegans* and *E. coli* luminescence was also studied (Fig. 3). Results show toxicity of the compound to *C. elegans* and indicate that an EC_{50} value for *n*-decyl aldehyde is less than 0.03%. *E. coli* cells proved to be far more tolerant to *n*-decyl-aldehyde. At concentrations which greatly reduced the luminescence of yeast or *C. elegans* greatly, the *E. coli* cells showed high levels of luciferase activity. Further exposure of the *E. coli* cells to 0.1% *n*-decyl-aldehyde only reduced luminescence to 91.5% of its maximum (data not shown).

Many aldehydes are known toxins and one theory for evolution of the *lux* system was as an aldehyde detoxification mechanism [17]. Prolonged exposure to *n*-decyl-aldehyde is toxic even to many bacterial species. The LucΔ *S. cerevisiae* strain, the luminescent nematodes and bacteria used for the toxicity bioassays, exploit a firefly luciferase system for quantifying the toxic effects of compounds through disruptions in the intracellular ATP pools [7,8]. The above firefly luciferase expressing organisms were applied to test the toxicity of *n*-decyl aldehyde as this compound is not required for the firefly luminescence reaction. The response of both eukaryotic strains (Fig. 3) indicates that it is *n*-decyl aldehyde itself that is toxic to them at very low concentrations. Given that the addition of *n*-decyl aldehyde, at concentrations greater than the EC_{50} values indicated by this study, is an essential requirement for quantification of bacterial luciferase activity, our results do not support the use of *lux* reporter genes for in vivo analyses in these model eukaryotes.

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