Biosensors and Bioelectronics 123 (2019) 1–6

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

O_2-requiring molecular reporters of gene expression for anaerobic microorganisms

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ARTICLE INFO

Keywords:
Bioluminescence
Gram (+)
Gram (-)
Luciferase
Arabinose induction

ABSTRACT

Many genetic reporter systems require molecular oxygen; therefore, the use of reporter genes to study molecular mechanisms in anaerobic microorganisms has been hampered by the lack of convenient reporting systems. We describe reporter gene whole cell-based biosensor systems based on luciferase genes and the associated oxygen-requiring enzymes. By using two different oxygen-dependent reporters, insect and bacterial luciferases, and two bacterial hosts, Gram (+) *Bifidobacterium longum* and Gram (-) *Escherichia coli*, we show that the enzymes can be used in gene expression studies of anaerobic bacteria. *E. coli,* a facultative anaerobe, was grown both in aerobic and anaerobic conditions with an arabinose-inducible expression system. We show that a short treatment time of few minutes in ambient atmosphere is sufficient to detect light emission from living cells that is directly proportional to the number of cells and to the inducer concentration. The induction levels were the same in both the aerobically and anaerobically cultured cells. Similar results were obtained in the case of *B. longum* cultured in anaerobic conditions.

1. Introduction

Anaerobic microbes are an important class of organisms because they can be used in a vast number of biotechnological applications, and these microbes have been used since ancient times in the production of food and feed, with wine and beverages being the most well-known products. Upon the origin of life on Earth approximately 3.9 billion years ago (Rizzotti, 2009), the conditions were anaerobic, and the first living organisms were heterotrophic. The abundance of sub-seafloor microbes is expected to be $35.5{\cdot}10^{29}$ cells, comprising 55–86% of Earth's prokaryotic biomass and 27–33% of Earth's living biomass, and most of these cells are anaerobic, methanogenic bacteria or archaea, which dominate in deep sea sediments (Whitman et al., 1998); therefore, they present a vast, nearly unknown reservoir of organisms that have not yet been studied at the gene expression level. Furthermore, anaerobic microorganisms have an enormous market potential in various biotechnological applications, such as anaerobic digestion in sewage treatment systems, bioenergy production (H_2), alcohols and methane bioprocesses, production of fermented food products and in metabolic engineering in cosmetics, prebiotics and medicines. Therefore, it is strongly justified to identify convenient and simple methods for studying gene expression in both Gram (+) and Gram (-) anaerobic microorganisms, and also fungi and yeasts.

Research with anaerobic organisms has lagged behind that with aerobic microorganisms, partly because there are too few powerful molecular tools for efficiently studying the intracellular mechanisms in physiology and biochemistry at the molecular level. Reporter genes are tools that are used in place of each target regulon in cases where gene expression and its regulation are studied (Daunert et al., 2000; Elad et al., 2008). The gene encoding ß-galactosidase has been the most widely used reporter in anaerobic research because its product can be easily measured by a simple colour reaction (Feustel et al., 2004). Green fluorescent protein (GFP) and its colour variants have been shown to be unsuitable reporters in anaerobic bacteria because their correct chromophore formation requires aerobic conditions (Hansen et al., 2001; Tsien, 1998). Drepper et al. (2007) showed that blue-light photoreceptors from *Bacillus subtilis* and *Pseudomonas aeruginosa* contain light-oxygen-voltage-sensing domains that can be engineered to work as fluorescent reporters for gene expression analysis under both aerobic and anaerobic conditions. Bioluminescent proteins require oxygen as a substrate in their light-emitting reaction. Reporter genes supporting bioluminescent or fluorescent detection technologies have several benefits over other approaches whose activities are detected by spectrophotometry, such as ß-galactosidase (Feustel et al., 2004) and alkaline phosphatase (Edwards et al., 2015), or radioactivity (chloramphenicol acetyltransferase), namely, high sensitivity and real-time

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https://doi.org/10.1016/j.bios.2018.09.066
Received 30 May 2018; Received in revised form 13 September 2018; Accepted 19 September 2018
Available online 20 September 2018

detection. Most of these approaches, with the exception of luciferases, require cell disruption as an extra step in activity measurements. In principle, luciferase reporters have the highest sensitivity because biological samples do not produce light of their own (except for light-emitting organisms), given that the measurement chamber is a black, light-tight box that blocks ambient light. The use fluorescent reporters suffers from cellular autofluorescence that is triggered upon excitation, thus lowering the sensitivity. Both bacterial and insect luciferases (Michelini et al., 2008) are widely used as molecular reporter systems, but their use in anaerobic applications is believed to be restricted by the requirement for oxygen in the light-emitting reaction.

There are few reports on the use of luciferase-based reporters in anaerobic microorganisms. However, there are no in-depth analyses of how they function. For instance, the first paper by Phillips-Jones (1993) described the use of the *Vibrio fischeri luxAB* genes for monitoring gene expression in anaerobically cultured *Clostridium perfringens*. Not until ten years later were the *luxAB* genes used to monitor the effects of antimicrobial agents against nonreplicating, anaerobically grown *Mycobacterium tuberculosis* (Cho et al., 2007). In a decade-old report, fusions of the mercury resistance operon (*mer*) with a promoterless *luxCDABE* operon from *Vibrio fischeri* (Selifonova et al., 1993) were used to study the effect of intracellular pH on the accumulation of trace concentrations in *Escherichia coli* in anaerobic conditions (Golding et al., 2008). In that study, as in the previous examples, the measurement of light emission from the luciferase reporters was measured by shaking the samples in aerobic conditions after growth in anaerobic conditions, and the experimental setups were not shown in detail. It was found that only the β-galactosidase reporter gene worked properly in a tumour hypoxia model when monitored with in vivo imaging (Cecic et al., 2007). This result shows that it is important to provide molecular oxygen to the cells expressing reporter gene products that require oxygen for catalysis.

The light emission reaction by bacterial luciferases has the following formula:

$$FMNH2 + O_2 + RCHO \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + h\nu\ (490\ \text{nm})$$

where RCHO is a long-chain aldehyde and RCOOH is the corresponding fatty acid. Light-emission can be obtained from cells containing the structural genes of the bacterial luciferase operon, *luxCDABE*, without any external additives.

The reaction catalysed by insect luciferases has the following formula:

$$ATP + O_2 + D{-}luciferin \xrightarrow{\text{luciferase}} AMP + PP_i + CO_2 + oxyluciferin + h\nu\ (560\ \text{nm})$$

Light-emission can be obtained from cells that are supplied with external D-luciferin.

Both types of luciferase reporter proteins require molecular oxygen for catalysis. We report here that by applying a short recovery step under aerobic atmosphere prior to bioluminescence measurements, bacterial and insect luciferases (biosensing elements) can be used as molecular reporters in anaerobically cultured *Escherichia coli* and *Bifidobacterium longum* model organisms. We also show that gene expression closely parallels the dose-response using induction of the arabinose-inducible promoter in both aerobic and anaerobic conditions.

2. Materials and methods

2.1. Construction of Sak-Lux plasmid for Escherichia coli expression

The construction of the arabinose-inducible, bacterial luciferase operon-containing plasmid is shown in the Supplementary Material.

2.2. Construction and cultivation of bifidobacteria harbouring the shuttle plasmid expressing the click beetle luciferase gene

Electrocompetent *Bifidobacterium longum* cells were prepared as described earlier (Guglielmetti et al., 2007). The human intestinal anaerobic bacterium *B. longum* NCC2705 was transformed by electroporation with a shuttle vector (pGBL8b) containing the insect luciferase gene from a click beetle (*Pyrophorus plagiophthalamus*) under the control of a strong phage T5 promoter. The bioluminescence reporter vector pGBL8b was constructed earlier (Guglielmetti et al., 2008). Electroporation was carried out with a MicroPulser Electroporator (Bio-Rad, Milano, Italy) set at 12.5 kV/cm and employing a 2-mm cuvette (the time constants obtained were between 3.9 and 4.2 ms). This recombinant bifidobacterial strain was cultivated in anaerobic conditions at 37 °C in MRS medium (Difco Laboratories Inc., Detroit, MI) supplemented with 0.05% cysteine-HCl and 10 μg/ml of chloramphenicol. Anaerobic conditions were created by the use of an anaerobic glove box (80% N_2, 10% CO_2, 10% H_2). The resulting clones displayed a bioluminescent phenotype. Light emission was studied in relation to anaerobic conditions.

2.3. Aerobic and anaerobic induction experiments for bacterial and insect luciferase reporter systems

pSak-Lux plasmid-containing *E. coli* XL1 was inoculated in 5 ml of L-broth with ampicillin (100 μg/ml) for overnight (O/N) incubation. O/N cells were inoculated into anaerobic tubes containing 10 ml LB at a 1% dilution and were grown to an optical density of 0.6. Further steps were carried out in an anaerobic glove box or in ambient oxygen concentrations.

500 μl aliquots of culture were transferred to Eppendorf tubes to which different concentrations of arabinose were added. One set of Eppendorf tubes was incubated at 37 °C in a glove box under anaerobic conditions, and another set was incubated at 37 °C, aerobically for 1 h. Thereafter, 200 μl of the samples was added to each well of the microtiter plate.

The recombinant *B. longum*/pGBL8b strain was grown and treated essentially the same as the *E. coli* strain containing the reporter construct. The measurement of the reporter activity in anaerobic conditions is described below.

Light emission was measured using Hidex Chameleon multilabel reader (Turku, Finland) from 96-well microtiter plates that had been kept either in ambient atmosphere (*E. coli*/Sak-Lux plasmid), or in an anaerobic chamber (*E. coli*/pSak-Lux plasmid and *B. longum* NCC2755/pGBL8b). For *B. longum*, 10 μl of 1 mM D-luciferin (Aboatox Oy, Turku, Finland) in 0.1 M Na-citrate buffer, pH 5.0, was pipetted into the plate wells to start the light emission reaction. The bioluminescence emission from the cultures was measured after aerobic treatment to quantify the gene expression. A short incubation time (from 5 min on) under ambient atmosphere was adequate for the bioluminescence detection from anaerobic cultures, and this bioluminescence closely paralleled that of aerobically grown *E. coli*/pSak-Lux cells. *B. longum*/pGBL8b cells from anaerobically grown cultures were measured essentially in the same way as with *E. coli*. The measurements were performed directly from unbroken, living cells.

2.4. Dilution tests

pSak-Lux plasmid-containing *E. coli* XL1 cells were inoculated into anaerobic tubes and incubated O/N at 150 rpm at 37 °C. The next day, 1% of the O/N culture was inoculated to another anaerobic tube and incubated at 37 °C/150 rpm until it reached an $O.D._{600\ nm}$ of 0.6. Serial dilutions were prepared from the 0.6 O.D. culture in different anaerobic tubes in the glove box (anaerobic chamber). The dilutions were as follows: 1; 0.1; 0.01; 0.001; 0.0001; 0.00001). A stock solution of 1% arabinose was added to each Eppendorf tube (containing 500 μl of the