17 Reporter Gene Technology in Soil Ecology; Detection of Bioavailability and Microbial Interactions

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17.1 Introduction

The overall purpose of many microbiological studies in soil is to describe the in situ activity of the indigenous soil bacteria. The aim of such studies is often to evaluate the physical and chemical conditions perceived by a bacterial cell while present in soil. Revealing this may be extremely difficult and sometimes even impossible, mainly because of the heterogeneity of the soil environment. Physical and chemical conditions such as water and oxygen content, and presence of nutrients and organic matter, may vary widely even within very small distances. Also organic compounds produced by the microorganisms in the soil, for instance inhibiting compounds or communication signals, may show a large spatial variation.

Using conventional analytical methods, the presence of anthropogenic compounds in a soil habitat can be measured. However, these methods require extraction of the compound from a certain amount of homogenised soil, and such methods tend to overestimate the availability of some compounds, which usually exist in an insoluble form in the soil (Chaudri et al. 1999). Additionally, the estimated concentration will be an average concentration of the compound in the soil due to the homogenisation. Microsensors are useful in relation to measurements in sediment microhabitats, as they are introduced into local areas of the sediment (Revsbech et al. 1980; Christensen et al. 1994; Lüdemann et al. 2000). They therefore may provide an indication of the conditions to which the bacteria are exposed in this bacterial habitat. However, microsensors are fragile, not suitable for use in heterogeneous environments, and may not always be sufficiently sensitive. Therefore, microsensors are less suited for use in soil. Thus, none of these methods are suitable tools to evaluate to which compounds the soil bacteria are actually exposed and whether those compounds are perceived and responded to by the bacterial cells. An alternative method for revealing this is by use of whole-cell biosensors.

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In this chapter, we present the most commonly used reporter genes and discuss the methods for detection of the reporter gene products, when expressed in soil. Additionally, the use of whole-cell biosensors in detection of bioavailability and microbial interactions will be discussed. We hope to provide the reader with an overview of reporter gene technology applied in soil studies and to illustrate the usefulness of whole-cell biosensors in studies of soil ecology.

17.2 Reporter Genes

A biosensor is a living organism, expressing a measurable product in the presence or absence of the investigated compound or condition. The measurable product is encoded by a reporter gene. Usually, the biosensor is genetically manipulated to contain the reporter gene, for instance in fusion to a regulated promoter; alternatively, the reporter gene is naturally present in the organism. An impact of the detected compound or condition on the biosensor is required for reporter gene expression; thus only conditions affecting the biosensor will be detected and responded to. Therefore, biosensors are very useful tools for detecting bioavailable concentrations of compounds in complex environments such as soil.

A wide range of whole-cell biosensors have been developed and used as tools to qualitatively and quantitatively identify the presence of different compounds or conditions in environmental samples (Hansen and Sørensen 2001). Biosensors have proven useful in detection of toxicity in samples, and therefore biosensor assays are potential candidates to replace conventional analytical methods. Moreover, biosensors have also been used in studies of microbial ecology and gene expression in complex environments (for a review, see Hansen and Sørensen 2001).

Reporter gene systems can be divided into several groups based on the nature of the product expressed. The most commonly used reporter genes in soil studies are *luxCDABE*, *lacZ*, *gfp* and *inaZ*. The properties of these reporter genes are described below and summarised in Table 17.1.

17.2.1 Reporter Genes Encoding Luciferases

Genes encoding light-emitting products are frequently used in biosensor constructs. Bacterial versions include the *luxCDABE* operon from *Vibrio fischeri*, the *luxCDABFE* operon from *Photobacterium leiognathi* and the luciferase coding *luxAB* genes from *Vibrio harveyi*. The *luxAB*-encoded

Table 17.1. Properties of reporter genes used in whole-cell biosensors (– = not applicable, x = little/low, xx = medium, xxx = much/high)

	luxCDABE	*lacZ*	*gfp*	*inaZ*
Sensitivity	xxx	xxx	xx	xxx
Oxygen requirement	yes	no	yes	no
Co-factor independency	x	xxx	xxx	xxx
Expression at low metabolic activity	x	xx	xx	xx
In situ detection	xx	–	xxx	–
Single-cell detection	x	x	xxx	–
Handling ease	xxx	x	xxx	xx
Cell disruption necessary	No	Yes	No	No
Low-cost	xx	xxx	x	xxx

luciferase is a heterodimer composed of two different subunits. It catalyses the oxidation of $FMNH_2$ and a long fatty acid aldehyde in the presence of molecular oxygen. The aldehyde is subsequently regenerated by a multienzyme reductase complex, which is encoded by *luxCDE* (Elasri and Miller 1998). Thus, no substrate addition is required when the *luxCDABE* operon is used and light can be measured in vivo.

The measurement of light is carried out very quickly in a luminometer (Tauriainen et al. 1999) or a scintillation counter (Selifonova et al. 1993). In many studies, fibre optic technology has been used to detect light emission from biosensors containing the *lux* gene (Heitzer et al. 1994; Corbisier 1997; Matrubutham et al. 1997). Typically, cells are immobilised in, for instance, an alginate gel which is placed on, or close to, optic fibres. By connecting the light-guiding optic fibres to a photomultiplier and a recorder, light emission from biosensor cells can easily be quantified.

The main advantage of the *lux* reporter system is that visualisation of the light from the luciferase can be carried out without disruption of the bacterial cells which makes *lux* genes suitable for rhizosphere studies (Kragelund et al. 1997; Jensen and Nybroe 1999). Even if only *luxAB* is used, the aldehyde (the substrate for the light reaction) can be added externally without disrupting the spatial distribution of the bacteria. This can prove particularly advantageous when monitoring distributions of biosensor bacteria in situ. Kragelund and coworkers used a *luxAB* biosensor responding to phosphate starvation, to report phosphate limitation in the rhizosphere of barley (Kragelund et al. 1997; Jensen and Nybroe 1999). A root-colonising bacterium, *Pseudomonas fluorescens*, was allowed to colonise the roots of barley in sterile soil. Thereafter, the roots were exposed to aldehyde vapours and examined using a photonic camera. The roots were brightly luminescent but, after subsequent addition of a phosphate buffer, luminescence

was repressed. This was done without disassociating the bacteria from the roots. The detection limits of advanced photographic detectors allow detection of luminescence originating from aggregates of a few bacteria or even of single cells (Rattray et al. 1995), even though the resolution needs improvement.

Eukaryotic versions of reporter genes which encode light production have also been used in biosensor constructs. These are the luciferase genes, *luc* genes, from the firefly (*Photinus pyralis*) or click beetle (*Pyrophorus plagiophthalamus*; Tauriainen et al. 1998, 1999; Willardson et al. 1998).

Luciferase-based biosensors are either used in situ or alternatively incubated in soil extracts or soil pore water followed by luminescence measurements (Chaudri et al. 1999; Hansen and Sørensen 2000; Porteous et al. 2000; Rasmussen et al. 2000; Vulkan et al. 2000; Flynn et al. 2003). When applying the luciferase-encoding genes as reporters in soil or soil extracts it is necessary to ensure that luminescence is not quenched by soil particles (Rasmussen et al. 2000). Additionally, it should be noted that production of light is an energy-demanding process, and therefore the light output is directly affected by the physiological state of the biosensor cells (Jansson 2003). This is especially important to consider in studies where the biosensor is inoculated in bulk soil for a longer period of time and may become energy deprived. In these cases, it could be advantageous to choose a reporter gene of which expression does not require a metabolic activity as high as that required for the luciferase-encoding reporter genes.

17.2.2 Reporter Genes Based on Chromogenic Detection

The *Escherichia coli lacZ* gene encoding β-galactosidase is also widely used as a reporter gene in biosensors. This reporter gene is extremely reliable which is mainly caused by the simplicity of the *lacZ* gene and its product, β-galactosidase, as well as by the fact that the *lacZ* gene is probably the best-studied reporter gene available. β-Galactosidase can be produced under most circumstances, i.e. it does not require oxygen as do both *luxCDABE* and *gfp* or any other special growth-dependent cofactors (like $FMNH_2$), and it also requires less metabolic activity than *luxCDABE*. The detection of β-galactosidase relies mainly on extraction of the protein from the biosensor cells and a subsequent β-galactosidase assay (Miller 1972). This requires more labour than the direct detection of *lux* and *gfp* products which are both based on photometric detection of emitted and fluorescent light, respectively. However, the β-galactosidase assay provides reliable quantitative responses and, unlike many other reporters, the detection of β-galactosidase does not require expensive equipment.

β-Galactosidase catalyses the reactions of a variety of substrates to different coloured products which are easily detectable. Additionally, some substrates can be converted into products which are oxidised at an electrode, resulting in electrochemical signals that can be measured (Biran et al. 2000).

The *lacZ* gene is not used as frequently in soil studies as the *lux* and *gfp* genes. The reason for this may be that the β-galactosidase measurement requires disruption of the cells and does not allow simple single-cell detection. However, by the use of β-galactosidase-specific fluorescent antibodies, single-cell analysis of *lacZ* expression in soil is possible by flow cytometry (Koch et al. 2001). Biosensors containing a *lacZ* reporter fusion can be inoculated in the soil followed by extraction and disruption of cells, and finally β-galactosidase measurement (Van Overbeek et al. 1997; Højberg et al. 1999; Biran et al. 2000).

Reporter genes like *xylE* (encoding catechol 2,3-dioxygenase), *phoA* (encoding alkaline phosphatase), *uidA* (encoding β-glucuronidase, the GUS reporter system) and *cat* (encoding chloramphenicol acetyl transferase) can be assayed with chromogenic substrates (Miller 1992). They are all potential reporter genes in bacterial biosensor constructs, but they are used less frequently than *lacZ*.

17.2.3 Reporter Genes Encoding Fluorescence

Many recent studies have used the expression of the *gfp* gene, encoding a green fluorescent protein (GFP), as a marker of bacteria introduced into different environments, including soil and rhizosphere. The *gfp* gene was isolated from the jellyfish *Aequorea victoria* and encodes a stable, green fluorescent protein (Chalfie et al. 1994). Detection of GFP requires only excitation by light (typically 395 or 488 nm depending on the GFP variant; Cormack et al. 1996; Errampalli et al. 1999) followed by measurement of emitted light. It is therefore not limited by the availability of substrates, like the detection of both β-galactosidase and the eukaryotic luciferases. Bacteria containing GFP can be detected at the single-cell level. The detection of single cells relies on techniques like epifluorescence microscopy (Joyner and Lindow 2000; Casavant et al. 2002, 2003) or flow cytometry (Hansen et al. 2001; Burmølle et al. 2003). Spatial information can be obtained at high resolution even from GFP-containing bacteria in environmental samples by using confocal laser scanning microscopy (CLSM; Bringhurst et al. 2001; Steidle et al. 2001). An example of visualisation of *gfp*-expressing biosensor cells at the single-cell level by CLSM is presented in Fig. 17.1.

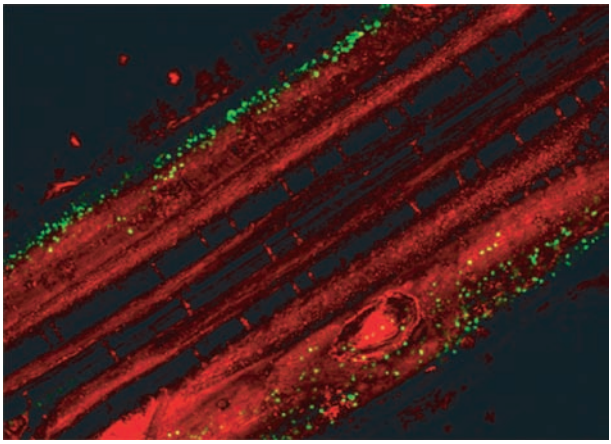

Fig. 17.1. Visualisation by confocal laser scanning microscopy of *gfp*-expressing biosensor cells on a wheat straw. A biosensor detecting bacterial communication signals, *N*-acylhomoserine lactones, was introduced onto the straw (Burmølle et al., unpubl. res.)

In recent years, *gfp* has been used more and more frequently as a reporter gene, especially in environmental studies. This is mainly due to the ability of GFP detection at the single-cell level, although this detection requires expensive and advanced equipment. However, single-cell detection provides a powerful tool for in situ studies of complex habitats such as rhizosphere (Bringhurst et al. 2001; Steidle et al. 2001) and bulk soil (Hansen et al. 2001; Burmølle et al. 2003). In such studies, *gfp*-based biosensors are usually incubated in bulk or rhizosphere soil and thereafter analysed for green fluorescence by one or several of the above-mentioned methods. Alternatively, *gfp*-expressing biosensors can be incubated in soil extracts and then be analysed by fluorescence measurements. However, in such applications, the sensitivity of the *gfp* gene is low compared to the *lux* and *lacZ* reporter systems.

The presence of oxygen is required for the GFP protein to fold properly and fluoresce (Tsien 1998). Thus, in oxygen-limited conditions, which occasionally are present in soil, GFP-expressing biosensors may not be suitable. In our laboratory, however, we have not experienced this feature of GFP to be a problem during applications of *gfp*-based biosensors in soil. Additionally, if the biosensor cells are handled in samples prior to analysis, for instance during extraction from the soil, the sample will become aerated and the GFP present in the cells will fold up and fluoresce.