

Lac as a marker gene to track microbes in the environment

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Introduction

The environmental use of microorganisms as a means to combat pests and diseases, to remediate contaminated land and act as bio-fertilisers and plant growth promoters has been a focus for research for many years. However, the successful application of living microbial inocula is dependent on a thorough understanding of the behaviour of the organisms involved in relation to the environment into which they are released [8]. In order to gain this type of understanding, a large variety of selective and semi-selective media have been developed that allow selective recovery of organisms from environmental samples. Such media make use of specific characteristics that are inherent to the organism under study. Such characteristics include, growth conditions (temperature, pH, etc), resistance to antibiotics and heavy metals, tolerance to osmotic pressure and/or expression of phenotypic characteristics such as fluorescent pigments, coloration and morphology. In this way, media have been designed for the isolation and quantification of many ecologically important bacteria [27]

Recent advances in recombinant technology have allowed the identification, characterisation and isolation of genes that encode for useful characteristics in relation to environmental applications. After isolation, and introduction into suitable vector systems, such genes can subsequently be introduced into a single recipient organism, allowing the creation of a genetically modified microorganism (GMM) with potentially 'improved' characteristics for environmental use. However, public concerns over the possible (negative) consequences of releasing functionally 'improved' organisms into the environment has increased the need for the development of sensitive detection techniques for microorganisms in environmental samples. The need to detect GMMs in the environment has created an impetus for the development of detection methods employing molecular techniques. Besides genes that encode for functional characteristics that are aimed to improve the environmental performance of a GMM, a variety of genes have been isolated from several organisms to facilitate detection of a particular GMM in the environment. Depending on the type of genetic marker employed, a GMM may be tracked phenotypically, relying on the expression of the marker gene by the GMM. Alternatively,

the presence of a marker gene can be identified by methods that do not rely on its expression [19]. In general, marker genes can be divided into three groups:

1. Short but unique oligonucleotide sequences that act as a genetic signature.
2. Genes that provide a selectable characteristic, such as resistance to an antibiotic, a heavy metal or ability to metabolise an unusual chemical.
3. Chromogenic markers that provide a colour change.

Chromogenic markers contain one or more genes whose presence in a microbial cell may be detected by an ability to produce a colour change in a substrate. Isolation of microorganisms on media supplemented with substrates that are capable of being transformed into coloured products has been used successfully to distinguish bacteria expressing the *xylE* gene, which encodes for catechol 2,3-dioxygenase [30] and those that carry the *lacZY* genes from *Escherichia coli*. The latter encode β -galactosidase (*lacZ*) and lactose permease (*lacY*) [14]. Other systems in this category are GUS (expression of β -glucuronidase), the *lux* operon from *Vibrio fischeri*, in which organisms are identified by their ability to bio-luminesce [28] and accumulation of GFP (Green Fluorescent protein) within cells that express the GFP genes from the jelly fish *Aequorea victoria* [5].

The *lac* operon

The *lac* operon of *E. coli* spans approximately 5300 base pairs and includes the *lacZ*, *lacY* and *lacA* genes in addition to the operator, promoter and transcription terminator regions. Since its description [18], the lactose operon has been a model system of great usefulness in biology. Study of this operon has touched on some significant questions in biology. For example, fundamental questions of the mechanisms involved in expressing genes were first studied in this system [18]. The discovery of the *lac* repressor and its binding to an operator site on the DNA was one of the first problems concerning protein-DNA interactions to be examined [25]. Studies of β -galactosidase in relation to *lacZ* mutants have been important in defining many aspects of gene-protein relationships. Studies with fragments of β -galactosidase also have served as a model system for investigating protein-protein interactions. The lactose permease, the product of the second structural gene, *lacY*, was the first membrane transport protein that was studied extensively. Many fundamental concepts of the transport of molecules into the cell were derived from these studies. It is therefore not surprising that structures of the lactose operon have been investigated intensively [3]. For example, Fowler and Zabin [15] reported the amino-acid sequence of β -galactosidase in 1978. When methods for determining DNA sequences became available, the DNA sequence of both the *lac* repressor and the control elements of the lactose operon was determined [3,16]. After the DNA sequence of *lacY* was confirmed [4], the DNA sequence of *lacZ* was determined [20] with the amino acid sequence of β -galactosidase as confirmation. The third component of the *lac* operon, *lacA*, was sequenced in 1985 and its amino-acid sequence determined [17]. The *lacA* gene encodes for

thiogalactosidetransacetylase and is thought to be involved in detoxification of thiogalactosides by the cell.

Procedures

Genetic marking of fluorescent Pseudomonas with lacZY

To be of use for tracking microbes in the environment, marker genes need to be rare in the release environment to allow distinction between the marked organism and indigenous populations on non-selective media. Alternatively, the isolation medium should be selective, not allowing growth of indigenous bacteria that express the phenotypic features encoded by the marker gene. All fluorescent pseudomonads isolated from the environment are unable to use lactose as a carbon source. This feature makes the genes that make up the *lac* operon ideal as genetic markers for this group of bacteria. Not only do these genes enable a recipient organism to utilise lactose as the sole carbon source, but the *lacZ* gene, which encodes for β -galactosidase, allows cleavage of the substrate X-gal (5-chloro-4-bromo-3-indolyl- β -D-galactopyranoside) into a bright blue product that is easily recognised [14]. To determine whether *lacZ* alone was sufficient for lactose utilisation, *lacY* was eliminated on broad host-range plasmids (pMON5002 and pMON5013) which were constructed to carry genes into recipient bacteria. Plasmid pMON5002 was restricted with EcoRI at the unique site in the *lacZ* coding sequence and with BglII, which cuts uniquely down stream from *lacY*. The incorporation of the *lacZ* gene alone did not confer the ability of transformed *Pseudomonas* cells to efficiently utilise lactose as a sole carbon source. The inclusion of *lacY* (lactose permease) enabled growth at a rate comparable to that observed on glucose. This implies that in *Pseudomonas*, as in *E.coli*, β -galactosidase remains a cytoplasmic protein, which does not gain access to sufficient lactose, without an active lactose transport mechanism. The *lacY* product, lactose permease, provides this transport mechanism [14].

Although different strategies can be employed to generate *lacZY* marked bacteria, the following might serve as an example of procedures employed by Barry [2] to obtain a *lacZY* marked *P. aureofaciens* that was used for release in the field [12]. Although the *E. coli lacZY*

genes expressed from different promoters on broad host-range plasmids are highly effective selectable markers for *Pseudomonas* [14], these genes are equally effective when delivered by a transposon Tn7-*lac* element into the bacterial chromosome. The advantage of Tn7 is that the transposition gene products function in *trans* and only 160 base pairs at each terminus are required to allow this transposition to occur. In addition, Tn7 inserts with high specificity integrate at high frequency into the chromosomes of many Gram-negative bacteria. Tn7 typically has only one insertion site per bacterial chromosome and is relatively rare in the environment [24]. Therefore, using the Tn7 based delivery system eliminates the need for screening through unwanted transposon mutants [22]. Originally the Tn7-*lac* element was composed of two unstable plasmids of different incompatibilities. Although the method is useful for generating *lac*-marked *Pseudomonas*, features that make this method unsuitable for common use are that any manipulation of the Tn7 element has to be done on a low copy plasmid not amenable to fast multiplication [2]. Also the Tn7-*lac* element itself contains a number of unknown regions of DNA that might originate from *E. coli* or from $\phi 80$ or λ at the end of the *lacA* gene. The first improvement involved the use of the smaller IncQ plasmids in the cloning and delivery system. Because of the decrease in size and the apparent broader host-range of IncQ plasmids, the double IncQ system can be used effectively in the cloning of genes and in the introduction of Tn7-*lac* elements into *Pseudomonas*. To further facilitate the cloning steps, and in particular the construction of more versatile and more widely applicable Tn7-*lac* elements, a small replicon was made by making a deletion of pUC8. A 500 bp fragment was cloned in this from into the *E. coli* chromosome containing the Tn7 insertion site. Into this *E. coli* strain the Tn7-*lac* element had been transposed and the bacterium contained a helper plasmid. From the progeny of this bacterium, a replicon of 2 kb with an 11 kb transposon was isolated. In this form the Tn7-*lac* element was easier to manipulate. Smaller cloning Tn7-*lac* elements were then constructed and their effectiveness for the expression of the *lac* genes determined. Mono-component Tn7-*lac* delivery systems were developed to expand the range of bacteria that may be marked with the *lac* genes. These 'suicide' delivery vectors, based on unstable IncQ replicons or on pBRS22, eliminate the need for antibiotic sensitivities in the target bacteria and for the replication

of the delivery replicon. The IncQ-based mono-component delivery system was used to mark a fluorescent pseudomonad (Ps. 3732RN) to create Ps 3732RNL11. The Tn7-*lac* element in Ps 3732RNL11 is composed of around 1700 bp of the termini of Tn7 and the *lacZY* genes (and a truncated *lacA* gene) promoted by the *iuc* operon promoter. The element used (Tn7-*lac*7117) contains a number of restriction sites to allow ease of cloning of additional genes, promoter replacements or substitution of *lac* with other selectable markers.

For applications in the environment, clearly stable integration of recombinant materials into the genome is desirable. Bailey et al. [1] successfully inserted two gene marker cassettes into the chromosome of a *P. fluorescens* isolate (SBW25). Given the potential for random and deleterious insertion, a strategy was adopted to facilitate detection whilst minimising the possibility of gene exchange and metabolic disruption. Two distinct chromosomal sites were therefore selected, approximately 1Mb apart on the chromosome, namely sites Ee and site -6-.

Site Ee was isolated from an EcoRI fragment from SBW25. The marker *lacZY* genes were inserted into a unique BglII site within the Ee site under the control of the *iucA* promoter isolated from pMON7117 [2]. This fragment was transferred onto a mobilisable suicide delivery plasmid and integrated into the chromosome of SBW25 at the Ee site by homologous recombination. Site -6- was also isolated on an EcoRI chromosomal fragment. A marker gene cassette containing *KanR* and *XylE* genes were inserted into the unique BglII site within the -6- site, with the integrating fragment being delivered by electroporation.

The transformation of *Pseudomonas* spp. using mobilisable suicide vectors as above or electroporation as in our laboratories using pMC1871 (Pharmacia) is often difficult and inefficient, requiring large quantities of transforming DNA and much subsequent screening. *Pseudomonas fluorescens* SBW25 appears to be more difficult in this regard than most other fluorescent *Pseudomonas* but even here methodologies have been developed for successful integration of genes.

The following might serve as a guide:

1. To provide biomass suitable for transformation, careful growth of bacteria to mid exponential phase is required, using cells at OD = 0.6 (550 nm).

2. After chilling on ice for 30 minutes, cells are pelleted by centrifugation in Falcon tubes (3K rpm for 3 minutes) followed by resuspension in 15% glycerol (V/V).
3. Cells from an initial culture volume of 250 ml are finally resuspended in 250 μ l of glycerol solution and stored on ice.
4. Purified plasmid DNA is obtained at a concentration of 1 mg/ml. Forty five μ l of chilled SBW25 suspension is mixed with 5 μ l (5mg) of plasmid DNA in a pre-chilled microfuge tube and stored on ice for 1 minute before being transferred to a pre-chilled electroporation cuvette (2 mm gap, Biorad).
5. Electroporation is carried out using a Biorad Gene Pulsar apparatus (settings: 2.5KV, 200 (25(F)).
6. Within one minute of applying the potential, the cell suspension is mixed with 500 μ l of SOB broth and gently mixed.
7. The contents of the cuvette is incubated at 30 °C for 4 hours before plating onto tetracycline-containing LB plates followed by incubation at 30 °C for 48 hours.
8. An initial screen of recombinants is made by selecting for a *TetR*, *KanR*, *lacZ*⁺ phenotypes. Recombinants carrying the newly introduced DNA are identified by using PCR analysis of DNA from isolates using primers homologous to regions of DNA that should be present within the newly inserted DNA.
9. After genotypic identification, functional assays for insertion of desired sequences are normally carried out. For example, using hplc or bioassay plates where the inserted genes are responsible antibiotic biosynthesis.

Integration of DNA of up to 7kb in length has been successfully achieved using this method, with predicted genotype and functionality observed.

Recovery of fluorescent pseudomonads expressing *lacZY* from environmental samples

Selective agar based media

Bacteria expressing the *lacZY* marker genes will be able to grow on mineral media such as M9 [26] amended with 1% (w/v) lactose [14].

Intrinsic resistance to antibiotics, such as rifampicin, might achieve further selection from the native microbial populations. This approach allows sensitive selection of up to 1–10 colony forming units (cfu) per g of non-sterile soil [13]. Problems arise when the natural soil populations have a significant proportion of bacteria that can use lactose as a carbon source and/or is resistant to the antibiotics used to select for the recombinant strain. For example, 1% of the culturable microbial community in a silty-loam field soil (Hamble series) taken from Littlehampton (W. Sussex, UK) was able to utilise lactose as the sole carbon source. A further 3.5% of the community was resistant to kanamycin incorporated at a level of 100mg/l, while 0.03% of the culturable bacterial community could utilise lactose and expressed resistance to kanamycin [11]. Clearly, minimal media such as M9 [26], amended with lactose and/or antibiotics are of little use for the selective recovery of recombinants in such situations. In this case, it was estimated that the detection limit of a triple marked *P. fluorescens* strain (SBW25EeZY-6KX, expressing the *lacZY* genes for lactose utilisation, the *aph1* gene for kanamycin resistance and the *xyIE* gene encoding for catechol 2,3 dioxygenase), on minimal medium [26] amended with 1% (w/v) lactose, 50 mg/l X-gal and 100 mg/l kanamycin, was around 10^3 cfu/g soil [11]. On roots, where bacterial numbers are one to two log units higher than in soil, detection would only be possible if the recombinant was present in concentrations $>10^4$ cfu/g rhizosphere soil. Consequently, a more sensitive method was required to isolate *lacZY* marked *Pseudomonas* cells from environmental samples. A growth medium, called P-1, developed for the selective recovery of *Pseudomonas* strains producing fluorescent pigment [21] provided the solution. This medium is made up as follows:

KH ₂ PO ₄	1.0 g
MgSO ₄ .7H ₂ O	0.5 g
KCl	0.2 g
NaNO ₃	5.0 g
Desoxycholate	1.0 g
Betaine	5.0 g
Agar	15 g
Distilled water	1 litre
pH	7.2–7.4

To obtain a clear medium, the ingredients are mixed thoroughly in a dry flask, before water is added. After mixing with water, the pH is adjusted using 1N NaOH. In our case, per flask, 5ml 1N NaOH is pipetted into a litre of medium to obtain the required pH. The medium is then autoclaved. When the medium has cooled sufficiently (40–45 °C), 50mg Xgal is added (1 ml stock solution of 50mg Xgal per ml DMSO). This medium will allow growth of both *lacZY* positive fluorescent *Pseudomonas* and indigenous *lacZY* deficient fluorescent *Pseudomonas*. The former will produce dark blue fluorescent colonies, while the latter will form white fluorescent colonies when viewed under long wave UV light. However, it has to be noted that the production of the blue pigment that results from the cleavage of X-gal will to some extent mask the fluorescence of the *lacZY* positive bacteria on agar media. The medium thus created enables phenotypic selection of the genetically marked *Pseudomonas*, from a background of indigenous fluorescent *Pseudomonas*. As indigenous fluorescent *Pseudomonas* are common (typically between 10^5 – 10^6 cfu/g soil), further selective agents have to be added to obtain a medium that is more selective. In the case of the triple marked *P. fluorescens* described before, addition of 100 mg kanamycin per litre medium, will only allow recombinants to grow. No doubt the same result can be obtained using other antibiotics to which the strain under investigation is resistant.

Surprisingly, good selective recovery can be obtained using P-1 medium from mashed root and leaf material that is colonised by fluorescent *Pseudomonas*, even though this material contains relative large quantities of carbohydrates and other substances that can be utilised by a variety of microorganisms. This indicates that the betaine used in the medium as a carbon source is probably not crucial for the observed selectivity for fluorescent pseudomonads. The more likely chemical in the medium that is responsible for *Pseudomonas* selectivity is desoxycholate. In fact, incorporation of desoxycholate into TSA (tryptic soy agar) at a rate of 1g/l seems to induce selectivity for *Pseudomonas* (De Leij, unpublished results). Therefore, the use of selective media that obtain their selectivity independent of the carbon source allows recovery of recombinant cells from environmental samples that are relatively rich in nutrients. Examples include compost, sewage sludge, plant material and foodstuff. This also means that selective media for *lacZY* marked bacteria that are based on the

incorporation of lactose as the sole carbon source are unlikely to remain selective when recovery is attempted from samples that contain alternative carbon sources.

Most Probable Number techniques involving lacZY

The extreme selectivity of P-1 medium amended with antibiotics also allows the use of enrichment methods involving larger quantities of soil. Instead of using a selective agar medium, a broth can be created using the same recipe without the agar. A 0.1% soil suspension in P-1 broth amended with kanamycin (100 mg/l) and X-gal (50 mg/l) will provide a clear medium, that will turn blue after 3–7 days incubation in shake culture at 25 °C when at least 1 recombinant cell is present [9]. In theory, recombinant cells can be detected in this medium up to any required sensitivity (for example, using one litre broth, 1 cell/10g of soil can be detected). In practice, dilution series are prepared in 10ml selective broth and after incubation in shake culture those dilutions that turn blue are scored [9]. Whereas it is difficult to see fluorescence of *lacZY* positive colonies on agar, exposure of broth that contains *lacZY* positive fluorescent *Pseudomonas* will fluoresce intensely when exposed to long wave UV light. Most probable number estimates are subsequently used to estimate the number of cells in the original sample. Most probable number estimates, however, are inherently variable (unless large numbers of replicates and small dilution steps are used). So unless the number of recombinants in the sample are below the detection limit of agar plating (ca 50 cfu/g sample) dilution plating will be the method of choice to estimate the number of recombinant cells in a sample. In the case of qualitative detection of recombinant cells in environmental samples, enrichment is the more sensitive approach.

Application of *lacZY* marker genes in ecological studies

Environmental fitness of LacZY marked organism

The use of marker genes, such as *lacZY*, offers clear advantages in providing an extra tool allowing selective recovery of marked organisms from environmental samples. However, marker genes offer no

advantage to the modified organism in terms of its competitive ability. It seems therefore logical to assume that the extra metabolic load of expressing these genes will put the organism at a distinct disadvantage compared to the non-modified parental strain. For example, Compeau et al. [7] found that rifampicin resistant mutants of *P. putida* were recovered from soil at lower rates compared to their non-modified parents. After only two days in soil there was more than a 10-fold difference in recovery of the two strains. This problem is claimed not to occur with fluorescent *Pseudomonas* that are marked using the Tn7-*lac* system [23]. Co-inoculation of the rhizosphere of maize with a Tn7-*lac* modified *P. aureofaciens* (strain L11) and its non-modified parent (strain RN) at different ratios did not give a significant difference in recovery of the two strains 2, 3, 4, 8 or 11 days later [23]. Similarly, Bailey et al. [1] found that no difference in the competitive ability of the triple marked *P. fluorescens* SBW25EeZY-6KX and its non-modified parent in the phytosphere of sugar beet. However, recent investigations indicate that effects on environmental fitness of *lacZY* modified strains depend on the environmental conditions into which the recombinant is released. Environments, such as the rhizosphere of pea, provide enough nutrients to mask any difference in metabolic load. In environments with a certain degree of nutrient stress, such as in soil and the rhizosphere of wheat, the extra metabolic load conferred by expressing *lacZY* reduces the environmental fitness of the marked strain significantly [10]. Other factors, such as disruption of existing genes due to insertion of the marker genes seem to play no significant role [10]. In summary it can be stated that the use of *lacZY* as a marker gene, is likely to result in a lower recovery rate compared with the parental strain when conditions are adverse. In situations where there is little nutrient stress, it is unlikely that detectable differences between parental and the recombinant strain will occur.

Relevance of cfu counts

The use of the *lacZY* marker lies in the fact that these genes allow easy identification of microbial colonies that express β -galactosidase activity on selective media into which X-gal is incorporated. This method of estimating viable recombinant cells in an environmental sample assumes therefore that:

- All recombinant cells present in a sample can be extracted
- Each viable cell that is extracted will result in a distinct blue colony
- Incubation conditions, determined by nutrients, time and temperature, will activate all the recombinant cells in a given sample to multiply and form a colony.

Clearly none of these assumptions can be taken for granted. For example, when microbial cells are incorporated into soil, invariably only 10% of the cells added to the soil can be recovered. This phenomenon is difficult to explain, as there are several factors that influence recovery. First of all it is possible that a large proportion of the cells added to a soil system will die and are therefore lost. Secondly, some cells might become firmly absorbed to soil particles. Thirdly, it is possible that the extraction procedure (soil mixing, whirly-mixing, etc.) will damage cells beyond recovery. And fourthly, cells might clump together, forming apparently a single colony, while in fact this colony has originated from more than one cell. The last factor touches on the second assumption that each viable cell will form a distinct blue colony. Clearly, a colony might originate from several cells, in which case the estimate of the recombinant population in soil will be an underestimation. Furthermore, cfu counts on agar media rely on the ability of cells to multiply. However, this might not always be the case. It is now generally accepted that many bacteria can enter a viable but non-culturable state [6]. Cells that have entered this state are not recoverable using traditional plating techniques. There is some evidence that *Pseudomonas* cells could enter such a state [29]. It was found that one year after introduction into agricultural drainage water *P. fluorescens* counts were consistently higher using immuno fluorescence (IF) than using cfu counts on agar. This indicated that a proportion of the *P. fluorescens* added became non-culturable. It was however not clear if these cells were simply not viable or that they were viable but non-culturable as the IF technique can not distinguish between the two states. Our own investigations with the triple marked *P. fluorescens* described above, indicate that colonies formed on P-1 medium amended with kanamycin and X-gal can be phenotypically very different. During times of environmental stress, instead of forming loosely packed colonies after approx. 2–3 days incubation at 25 °C, colonies appeared after more than 10 days incubation. The morphology of those colonies was entirely different from those formed by

non-stressed cells. The colonies were small, very dark blue and the cells in the colony were very tightly packed (De Leij, unpublished observations). These observations indicate that prevailing environmental conditions can induce physiological changes in *Pseudomonas* cells that will affect their recovery on agar media.

Conclusions

In summary it can be stated that:

1. *LacZY* provides a convenient, safe, well-described marker system for bacterial species that do not possess these genes.
2. Incorporation of *lacZY* into the bacterial genome might lead to a slight reduction of environmental fitness when cells are moderately stressed.
3. Because members of the indigenous microbial community commonly express *lacZY* themselves, selection procedures based on the *lacZY* should be combined with suitable selective media.
4. Results obtained from cfu estimates almost certainly under-estimates the true viable recombinant population in the environment.

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- MMEM-6.01/1199**

<http://www.springer.com/978-1-4020-2176-3>

Molecular Microbial Ecology Manual

Kowalchuk, G.A.; de Bruijn, F.; Head, I.M.; Van der Zijpp, A.J.; van Elsas, J.D. (Eds.)

2004, XVI, 1778 p. In 2 volumes, not available separately., Hardcover

ISBN: 978-1-4020-2176-3