Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations

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Abstract

An integration vector was constructed to allow introduction of the gfp gene into the chromosomes of Gram-positive bacteria. Integration depends on homologous recombination between a short 458-nt sequence of the tet(M) gene in the vector and a copy of Tn916 in the host chromosome. Strains of Lactococcus lactis IL1403, Enterococcus faecalis JH2-SS, and Streptococcus gordonii DL1 stably marked with single chromosomal copies of the gfp were readily visualised by epifluorescence microscopy. The marked L. lactis strain survived poorly in a continuous culture system inoculated with human faecal flora, while the laboratory E. faecalis strain was lost at approximately the dilution rate of the fermenter. © 2000 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The green fluorescent protein gene (gfp) has been exploited in a wide range of applications as a reporter and marker in both prokaryotic and eukaryotic cells [1], and its potential is still being explored [2]. Our interest is in using GFP to track marked bacteria in complex ecosystems, such as the human gut, to obtain information on bacterial localisation, colonisation and gene transfer using either in vitro simulations or contained conditions in vivo. This may be of particular value in assessing the fate of food-borne pathogens, probiotics and genetically modified bacteria that might be used in food or animal feed products.

Previous work showed that although the GFP protein requires exposure to oxygen for fluorescence [2], gfp-marked strains of Escherichia coli or of Gram-positive bacteria could be identified after selective plating in air following anaerobic incubation in gut contents [3]. The instability of plasmid-marked constructs used in Gram-positive hosts in the absence of antibiotic selection, however, meant that it was not possible to distinguish between plasmid loss and loss of the introduced strain from the system. We report here a general method for introducing the gfp gene into the chromosome of Gram-positive bacteria, by homologous recombination into the tet(M) gene of the broad host range transposon Tn916. Expression of single chromosomal copies of the gfp was readily detectable by epifluorescence microscopy and the integrated genes were stably maintained in the absence of selection pressure. The survival of the marked bacteria was monitored in a complex simulated gut ecosystem.

2. Materials and methods

2.1. Bacterial strains and media

E. coli V850 (Em®) and Enterococcus faecalis V1114 cells were kind gifts from F. Macrina, while Streptococcus bovis JB1 and S. gordonii DL1 (previously S. sanguis Chal- lis) were kindly donated by T.R. Whitehead. The plasmid-free derivative of Lactococcus lactis, IL1403, was donated by Y. Duval-Ilhah and the strains of E. faecalis JH2-SS and CG110::Tn916 were obtained from A. Salyers. E. coli XLI-Blue cells (Stratagene) were grown in Luria broth.
(LB) and *E. coli* V850 in LB supplemented with 0.04% thiamine and 0.01% methionine [4] both at 37°C. *Lactococcus* spp., *Enterococcus* spp. and *Streptococcus* spp. were grown in GM17 broth (Difco M17 broth supplemented with 0.5% w/v glucose) at 37°C (30°C for *L. lactis*) unless otherwise stated.

2.2. DNA purification

Plasmids were purified from *E. coli* cultures using the alkaline lysis method [5]. Plasmids from *Lactococcus* spp. and *Streptococcus* spp. were isolated following one of two protocols [6,7]. Chromosomal DNA was purified using a small-scale version of the method of Flint et al. [8]. Other DNA manipulations including restriction digestions, vector ligations, Southern transfers and random-primed $^{32}$P labelling and hybridisations followed standard procedures [9].

2.3. PCR amplification

The presence of *gfp* sequences was confirmed by PCR as described previously [3]. Primers for the tet(M) gene corresponded to positions 249–267 and 688–707 respectively [10] and incorporated KpnI restriction sites at the 5' ends (forward primer: 5'TAGGTACCAGCTATGGTATGCAGGA-3' and reverse primer 5'GAGGTACCGGATCAGTTTCTACCTTCTG-3'). PCR amplification of *L. lactis* 16S ribosomal DNA utilised the universal eubacterial T primer [11] and a primer specific for lactococci ([12]; 5'GATGAAGATTGGTGCCTGCA-3').

2.4. Bacterial transformation and conjugation

Competent *E. coli* cells were prepared using the CaCl$_2$ method [9] and *E. coli* V850 cells containing pTRW-based plasmids were selected on 75 µg ml$^{-1}$ erythromycin (Em). Electroporation of *E. faecalis* CG110::Tn916 involved a modified version of the method of Cruz-Rodz and Gilmore [13]. A 40-ml culture of *E. faecalis* was grown in medium containing 8% glycine for 21 h, and cells were harvested and washed as described. Electroporation was carried out using a Bio-Rad Gene pulser with settings of 2.5 kV; 25 µF; and 200 Ω. Pulse times were consistently 6.6 ms. Following electroporation cells were resuspended in SM17MC medium [13] lacking glycine and incubated at 37°C for 2 h before plating out dilutions on selective media. Transformation of *S. gordonii* DL1 strains was carried out as described previously [14]. The *gfp*-containing plasmid was introduced into a wider range of Gram-positive hosts by conjugative mobilisation using a filter-mating method [15] with overnight cultures of parent cells.

2.5. GFP fluorescence

The fluorescence of GFP-expressing cells was assessed by eye using a hand-held BlakRay light (UVP model UVL-56; emitting longwave UV at 366 nm) to examine colonies, or using a fluorescence microscope (Olympus model BX50) fitted with an FITC filter set as described previously [3].

2.6. Fermenter studies

A single-stage continuous-flow fermenter was used as described previously [3]. The fermenter was operated anaerobically at 37°C by maintaining the growth culture under an atmosphere of oxygen-free nitrogen (200 ml min$^{-1}$), and using anaerobic medium maintained by continually bubbling through oxygen-free CO$_2$ (200 ml min$^{-1}$) which was allowed to equilibrate for 24 h before being pumped into the fermenter vessel. The operating volume of the vessel was 0.9 l, and the dilution rate was 0.036 h$^{-1}$. The vessel was inoculated with a 20% faecal suspension from a healthy individual, prepared in anaerobic 1 M sodium phosphate buffer, pH 7.2. This was allowed to stabilise for 96 h before adding the first bacterial inoculum on day 5, the survival of which was monitored for 7 days before the second inoculation. Chromosomally marked *L. lactis* and *E. faecalis* cells were cultured overnight anaerobically in the presence of Em (10 µg ml$^{-1}$), washed twice in 1/4 strength Ringer solution (Oxoid) and resuspended in 25 ml fermenter medium, before adding on separate occasions to the culture vessel. Total aerobe and anaerobe counts were made daily in triplicate on either nutrient agar plates (Difco) or M2GCS roll tubes [16] respectively. *L. lactis* and *E. faecalis* were specifically enumerated on M17 plates (Difco) containing Em (10 µg ml$^{-1}$) and rifampicin (Rif, 100 µg ml$^{-1}$), confirmed to give no background growth with the normal flora alone. Bacterial counts were made after growth for 48 h at 37°C (aerobes and *E. faecalis*) or 38°C (anaerobes).

3. Results and discussion

3.1. Construction of the integration vector

A pBluescript-based vector (pKPSPgfp) containing the *gfp* gene expressed from a constitutive lactococcal P32 promoter was available from previous work [3]. This construct was modified to provide an integration vector for Gram-positive hosts as follows. Firstly an erythromycin resistance gene suitable for selection in Gram-positive bacteria was excised from the pTRW10 shuttle vector [17] and cloned into pKPSPgfp. A 458-bp fragment of the tet(M) gene amplified by PCR was cloned into the resulting vector, utilising KpnI restriction sites incorporated in the primers. This formed the construct pKPSPgfp-int (Fig. 1), which is unable to replicate in Gram-positive bacteria, but contains a marker selectable in Gram-positive bacteria and a site for homologous recombination into the genome.
3.2. Chromosomal integration into Gram-positive bacteria

pKPSPgfp-int was introduced into E. faecalis CG110::Tn916 by electroporation, and transformants were selected on medium containing erythromycin. The transformation frequency was 25 transformants \( \times 10^3 \) per \( \mu \)g DNA. All of the transformants (E. faecalis Tn916/gfp) retained resistance to tetracycline, which was ascribed to the fact that E. faecalis strains frequently contain up to six copies of Tn916 [18]. Plasmid DNA could not be isolated from transformants and the presence of the gfp sequence in the chromosome was confirmed by PCR. Southern hybridisation of chromosomal DNA purified from transformed E. faecalis strains to a \( 32^P \)-labelled gfp PCR product confirmed chromosomal integration of the vector (Fig. 2). In some cases tandem copies of the gfp construct had inserted into the chromosome (Fig. 2 lanes 3–5). This was verified by comparing the hybridisation patterns of HindIII- and EcoRI-digested chromosomal DNA (not shown). The relative intensities of the bands obtained implied that transformant 5 contained two tandem copies of the construct while transformants 3 and 4 contained more than two copies, integrated into the same site on the chromosome (Fig. 2). Chromosomally marked E. faecalis cells were fluorescent when examined by epifluorescence microscopy, indicating that the presence of even a single copy of the gfp gene was sufficient for visualisation. The presence of an extra gfp copy did not significantly enhance visible fluorescence.

pKPSPgfp-int was also used to transform S. gordonii DL1 cells [14]. The conjugative transposon Tn916 was initially introduced into S. gordonii DL1 by transformation with the plasmid pAM120 [19], and the chromosomal integration vector (pKPSPgfp-int) was subsequently transformed into S. gordonii Tn916 with transformation efficiencies of \( 10^{-2} \) transformants per recipient cell. Chromosomal integration was confirmed and cells also demonstrated detectable fluorescence.

Since the conjugative functions of Tn916 should not have been interrupted by the insertion events the mobility of the transposon was assessed. Filter matings between S. gordonii Tn916/gfp and a rifampicin-resistant mutant of L. lactis IL1403 demonstrated that the Tn916/gfp construct was still mobile and gave conjugation frequencies of \( 1.5 \times 10^{-7} \) per recipient. Transconjugants of L. lactis (Rif\(^R\), Em\(^R\)) fluoresced, and it was confirmed that the colonies were not spontaneous Rif\(^R\) mutants of S. gordonii by PCR amplification using a primer combination specific for L. lactis 16S rDNA.

These results show that a relatively short region of homologous DNA (458 nt) is sufficient for chromosomal integration and subsequent expression of adjacent genes, and that sequences of whole plasmids can be stably integrated into the chromosome. The inclusion of extra DNA sequences into Tn916 does not prevent onward conjugative transfer of the transposon.

3.3. Marker stability

In order to determine the stability of the chromosomal marker E. faecalis Tn916/gfp and L. lactis Tn916/gfp cells were grown in the absence of antibiotic for 30 generations by serial subculturing. After this time the proportion of colonies retaining antibiotic resistance was calculated by comparing the ability of random colonies grown without

![Fig. 2. Southern blot illustrating the chromosomal integration of the gfp gene into E. faecalis following electroporation. Chromosomal DNA (1 \( \mu \)g) was digested with EcoRI and hybridised under stringent conditions (65°C) to a gfp-PCR product probe. Lanes 1–7: E. faecalis chromosomal transformants; lane 8: E. faecalis (pKPSPgfp); lane 9: control untransformed E. faecalis DNA.](image-url)
selection to subsequently grow in the presence or absence of antibiotic. All of the 100 colonies picked in each case grew in the presence of Em and, as expected, all were fluorescent. These results indicate that unlike plasmid-encoded gfp, the chromosomal marker is stably maintained in these strains in the absence of antibiotic selection.

3.4. Survival of chromosomally marked strains in a simulated gut ecosystem

Previous work showed that it was feasible to use the gfp marker to track strains of plasmid-marked bacteria against a background of human faecal flora, but it was not possible to differentiate between plasmid loss from the bacteria and loss of bacteria from the fermenter system [3]. Here, the survival of two chromosomally marked strains was followed under conditions designed to simulate the human colon. An anaerobic continuous-flow fermenter containing a stable population of human faecal bacteria was inoculated with L. lactis Tn916/gfp. The viable count of L. lactis monitored daily declines from $1 \times 10^9$ to $5 \times 10^3$ in 48 h, which is more rapid than the washout rate (Fig. 3) indicating that the cells are dying. This poor survival presumably reflects the fact that L. lactis is not a native gut bacterium and is therefore relatively intolerant of gut conditions [20]. However, following the rapid decline in numbers, a small population of marked L. lactis persisted after 3 days at a level of $\sim 1.2 \times 10^5$ at least until day 12 when E. faecalis Tn916/gfp was added. This tail population could have arisen either from gene transfer to native gut bacteria, or from the development of RifR, EmR L. lactis more tolerant of gut conditions. The E. faecalis inoculant survived rather better although it did not become established, the decline in numbers ($1 \times 10^9$ to $5 \times 10^3$ in 8 days) following the washout curve. E. faecalis is a natural gut inhabitant and the lack of establishment may be due to an absence of appropriate environmental niches or biofilms in the fermenter system, or to the fact that a laboratory strain was used as the inoculant. The detection of possible onward gene transfer involving the gfp would be greatly facilitated using FACS (fluorescence-activated cell sorting), and is currently being investigated.

3.5. Concluding remarks

The chromosomal integration method employed here, which does not interrupt the transfer functions of Tn916, provides a general method by which any bacterial species capable of hosting Tn916 can be readily marked with gfp, using conjugal transfer methods. Since Tn916 has a broad host range, including both Gram-positive and Gram-negative bacterial species [21], this could be a powerful marking tool. This work also shows that a single, chromosomally integrated copy of gfp is visibly detectable in various lactic acid bacteria. The marker gene was stably integrated following homologous recombination through a single 458-nt piece of DNA, and detection was robust enough to monitor bacterial populations against a large complex background of unmarked bacteria over a period of at least 17 days. GFP marking was instrumental here in tracking the fate of single L. lactis and E. faecalis strains in a simulation of the human large intestinal flora. The observation that L. lactis is rapidly eliminated agrees with previous findings [20] and it is thus unlikely that L. lactis used as a food additive could establish in the gut. The nature of the ‘tail’ populations detected here for both L. lactis and E. faecalis is the subject of continuing investigations to establish whether they represent products of gene transfer or of adaptation of the input strains.

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