

Construction of a green-fluorescent protein-based, insertion-inactivation shuttle vector for lactic acid bacteria and *Escherichia coli*

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Introduction

Streptococcus thermophilus is an important industrial microorganism used in the manufacturing of fermented dairy products such as yogurt and cheese. Genetic engineering approaches could be used to effect strain improvement of this bacterium by conferring new or improved metabolic capability. To this end, various cloning and expression vectors have been constructed (Mercenier, 1990; Solaiman *et al.*, 1992; Solaiman and Somkuti, 1993). Most of the existing vectors, however, contain antibiotic resistance genes as selection markers, and are thus undesirable in 'food-grade' fermentation processes. Various non-antibiotic based genetic systems have been explored as potential 'food-grade' selection markers. These include genes for sugar metabolism (Mollet *et al.*, 1993; Lin *et al.*, 1996), nisin resistance (McIntyre and Harlander, 1993), and peptidase (Leenhouts *et al.* 1991). In this communication, we describe the use of a green fluorescent protein gene as a non-antibiotic based selection marker in the construction of a shuttle vector for *Escherichia coli* and *S. thermophilus* and possibly other lactic acid bacteria.

Materials and methods

Bacterial strains, plasmids, and growth conditions

E. coli DH5' and DM1 (*dam*, *dcm*) was obtained from BRL Life Technologies (Gaithersburg, MD). *S. thermophilus* ST128 was from our laboratory collection. *E. coli* was grown in Luria-Bertani (LB) medium, and ST128

was cultured in a tryptone-yeast extract-lactose broth (Somkuti and Steinberg, 1986). Selection of *E. coli* transformants was performed in a medium containing ampicillin (100 g/ml), chloramphenicol (15–35 g/ml) and/or erythromycin (200 g/ml). Growth medium containing erythromycin (10–15: g/ml) and/or chloramphenicol (5 g/ml) was used to identify *S. thermophilus* clones. Vectors pUC18/19 were from BRL Life Technologies. pGFP was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). pER8 is a cryptic plasmid found in *S. thermophilus* ST108 (Somkuti and Steinberg, 1986).

Molecular cloning procedures

Enzymes used in the construction of recombinant plasmids, such as restriction endonucleases, DNA-modifying enzymes and T4 DNA ligase, were variously obtained from BRL Life Technologies and New England BioLabs (Beverly, MA). A heat-shock protocol was used to transform competent *E. coli* DH5' (BRL Life Technologies). *S. thermophilus* was electrotransformed as previously described (Somkuti and Steinberg, 1988). Plasmids were purified from *E. coli* by using QIAGEN Midi or Maxi kits (QIAGEN Inc., Chatsworth, CA), and from *S. thermophilus* by CsCl ultracentrifugation (Somkuti and Steinberg, 1986). DNA was analyzed by agarose gel electrophoresis in TBE buffer system (0.089 M Tris base, 0.089 M boric acid, 0.002 M Na-EDTA). When needed, DNA fragments were electroeluted from agarose gel by using a SixPac GE200 Eluter (Hoefer Scientific Instrument, San Francisco, CA). Further purification and/or concentration of nucleic acid samples was performed with Elutip-d columns (Schleicher & Schuell, Keene, NH).

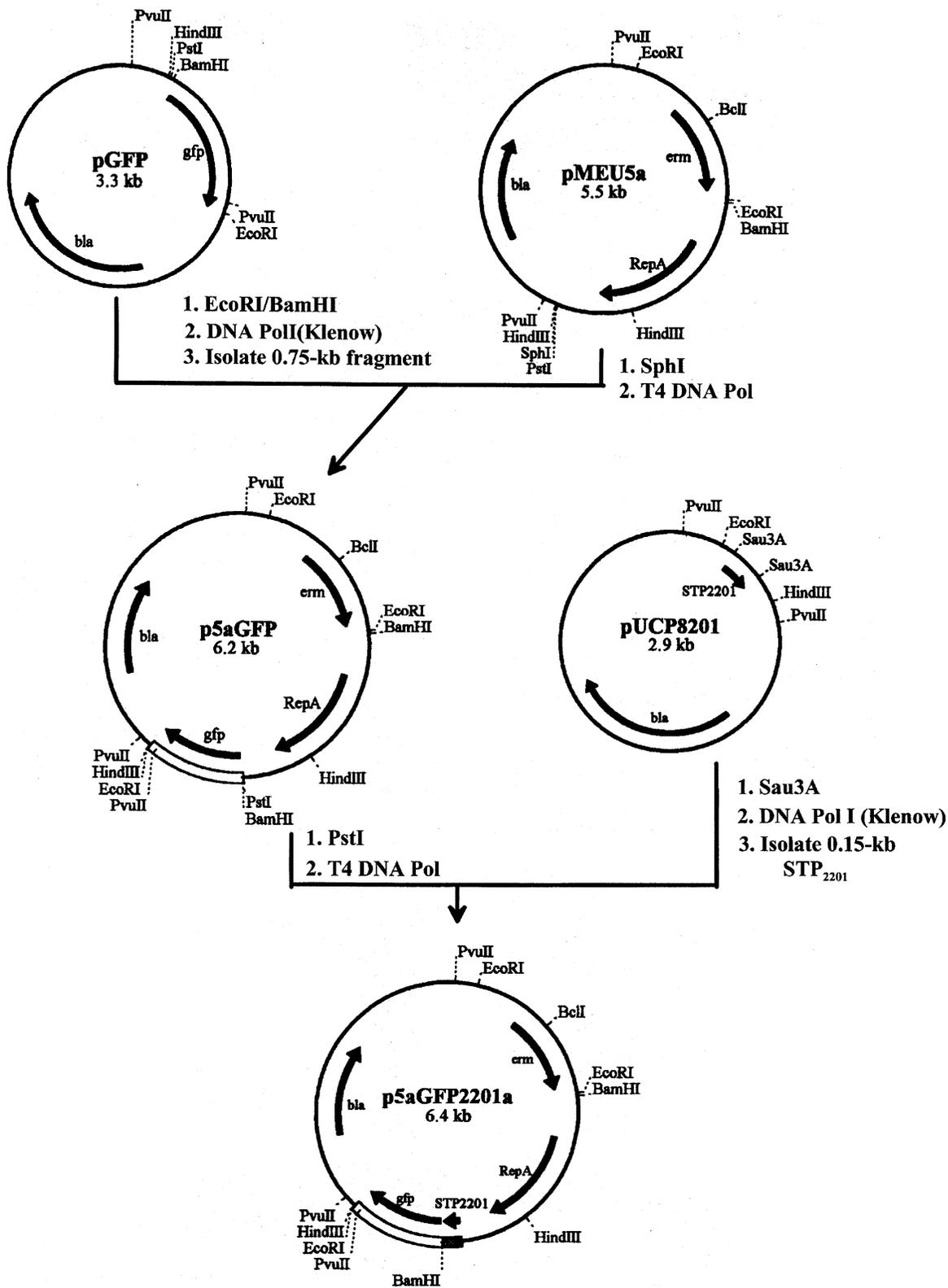


Figure 1 The Construction of p5aGFP2201a. For simplicity, not all restriction sites for any particular endonuclease were shown on the plasmid maps.

Results and discussion

Construction of p5aGFP2201a

The schematic for the construction of p5aGFP2201a is shown in Fig. 1. An *S. thermophilus*-*E. coli* shuttle vector, pMEU5a, was used as the building block (Solaiman and Somkuti, 1993). This vector is composed of three elements. These are: 1). *E. coli* pUC18 vector for plasmid propagation in the Gram-negative host and for transformant selection with ampicillin, 2). the *StyI/HinfI* (1.7-kb) fragment of pER8, which contains the replication function needed for plasmid maintenance in *S. thermophilus* and other Gram-positive bacteria, and 3). an *erm* (erythromycin-resistance gene)-containing DNA

segment from *S. chromogene* plasmid pPV141 (Somkuti *et al.*, 1997), which allows for selection of *S. thermophilus* transformants. Since *bla* is not operational in *S. thermophilus* (Solaiman *et al.*, 1992), pMEU5a-transformed ST could only be selected by erythromycin challenge. A second selectable marker that is functional in ST is thus required for the purpose of transformant identification based on insertional inactivation. The *Aequorea victoria* green fluorescent protein gene (*gfp*) was chosen as the second marker because it is not an antibiotic-resistance determinant and its expression does not require exogenous substrate nor co-factor (Prasher, 1995; Yang *et al.*, 1996). As shown in Fig. 1, the *gfp*

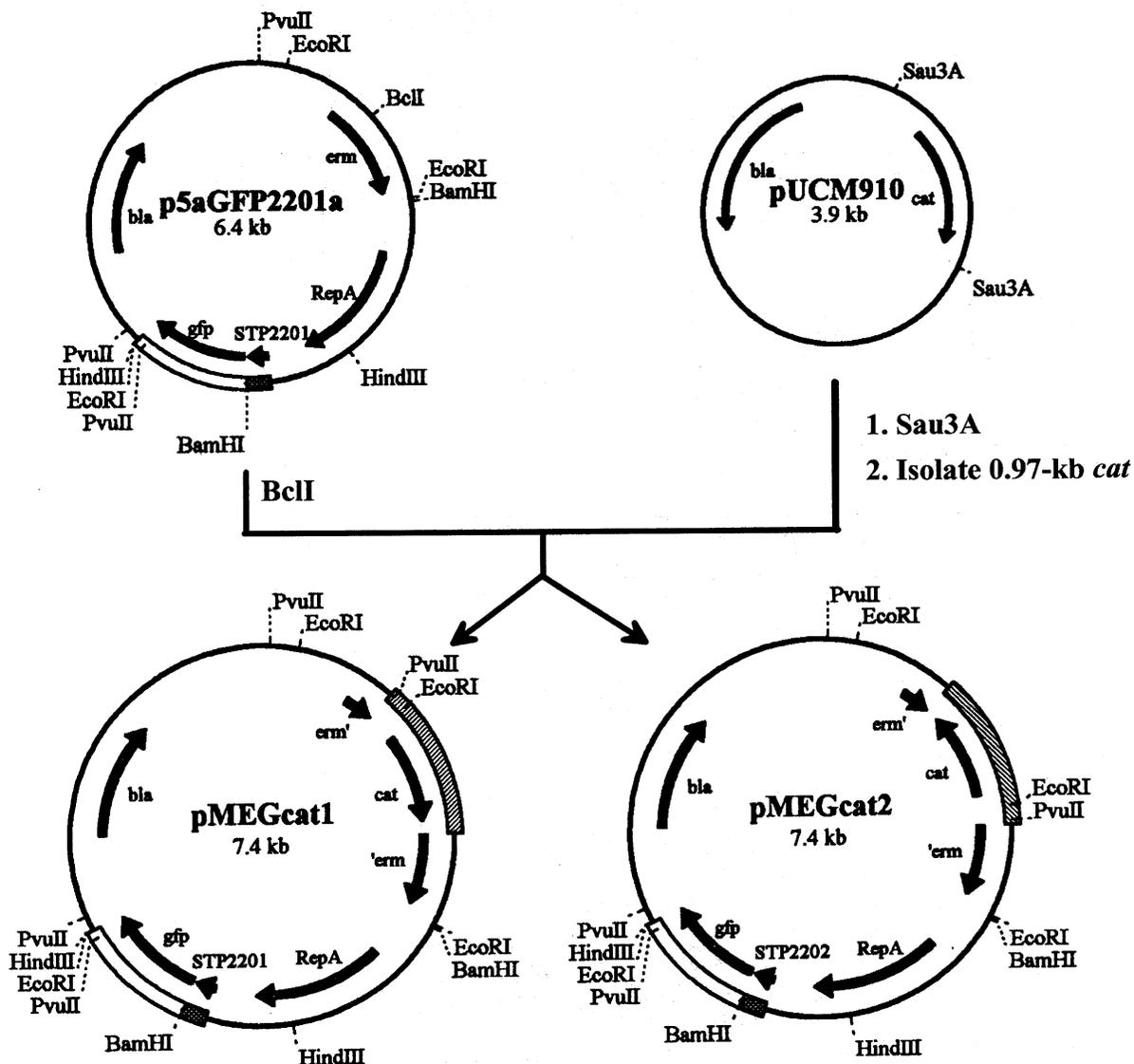


Figure 2 The Insertion of *cat* into p5aGFP2201a. Not all restriction sites for any particular endonuclease were shown on the plasmid maps.

gene was excised from pGFP plasmid and spliced into a unique *Sph*I site of pMEU5a to yield p5aGFP.

Since the isolated *gfp* does not contain a promoter, we next cloned an *S. thermophilus* promoter, ST_{P2201}, at the 5'-end of the gene. ST_{P2201} is a chromosomal promoter that was originally isolated as a *Sau*3A fragment from *S. thermophilus* ST128 and was shown to be active in *E. coli* and *S. thermophilus* (Solaiman and Somkuti, 1995; Somkuti and Solaiman, 1997). It was later subcloned into pUC18 by ligating an *Eco*RI/*Hind*III fragment of a pEU5a2201a plasmid (Solaiman and Somkuti, 1995) to the corresponding sites of the vector to produce pUCP8201. The ca.150-bp promoter was cut out from pUCP8201 and spliced into the *Pst*I site of p5aGFP by blunt-end ligation reaction. p5aGFP2201a in which the promoter is aligned with the *gfp* gene, was isolated from *E. coli* transformants that exhibited green fluorescence under UV illumination. This plasmid construct was introduced into *S. thermophilus* by electroporation at a transformation frequency of 8×10^2 colony-forming-unit (cfu)/ μ g DNA, and was shown to confer fluorescent phenotype to this Gram-positive host.

Use of p5aGFP2201a as insertional inactivation vector

Disruption of a marker by the insertion of a cloned gene forms the basis for insertional inactivation selection. Using a staphylococcal chloramphenicol acetyltransferase (*cat*) gene as a model, we demonstrated that p5aGFP2201a could serve as an insertional inactivation vector in which the unique *Bcl*I site of the *erm* gene provides for the splice site. p5aGFP2201a was propagated in *E. coli* DM1 (*dam*, *dcm*) strain to render the *Bcl*I site cleavable. The source of the *cat* gene was a pUCM910 plasmid which contains the chloramphenicol-resistance gene in a pUC19 vector (Solaiman and Somkuti, 1993). The *cat* gene was excised by *Sau*3A digestion, and was spliced into the *Bcl*I site of p5aGFP2201a by cohesive end ligation (Fig. 2). Recombination plasmids (pMEG*cat*1 and pMEG*cat*2) with the *cat* gene incorporated in the two possible orientation were isolated from *E. coli* transformants. In a random mini-plasmid screening, the two plasmids were recovered at approximately the same frequency from the transformants, indicating that the orientation of the *cat* gene did not affect plasmid transformation and maintenance in *E. coli*. Both the *gfp* and the cloned *cat* gene were fully expressed in this Gram-negative host, as evidenced by its fluorescent appearance and its resistance to chloramphenicol. Since the *erm* gene was clearly fragmented, it was presumed to be inactive and was not tested further.

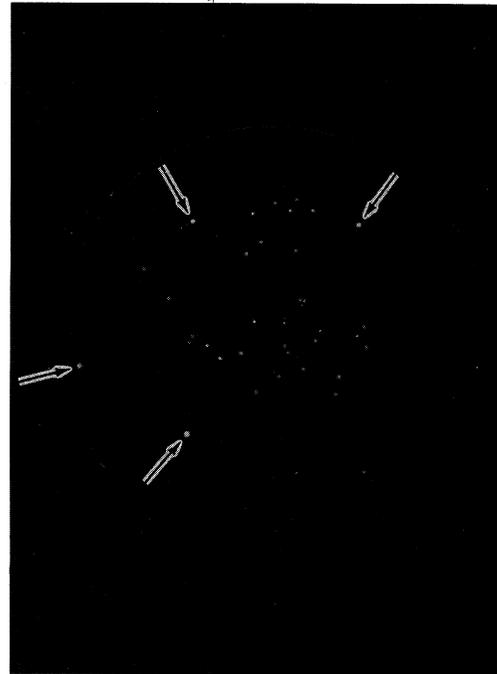


Figure 3 *S. thermophilus* transformants containing pMEG*cat*1. Plates were photographed under UV illumination.

The two pMEG*cat* plasmids were introduced into *S. thermophilus* ST128 by electrotransformation. pMEG*cat*1-transformed cells were recovered at a frequency of 4.2×10^2 cfu/ μ g DNA when selected on chloramphenicol-containing medium. All transformants exhibited fluorescent phenotype (Fig. 3). However, repeated attempts to electrotransform *S. thermophilus* with pMEG*cat*2 did not produce any chloramphenicol resistant transformants, suggesting that the inserted *cat* gene was not properly expressed. Since the *cat* gene in pMEG*cat*2 is positioned counter to the orientation of all the other genes in the plasmid, a 'collision effect' similar to that suggested for pauses in DNA replication might be in effect to limit its expression (Bierne and Michel, 1994). It should be noted that in this demonstration, clone selection was performed based on the activity of the inserted *cat* gene for convenience. In all other insertional inactivation experiments, however, the desired clones will actually be selected based on fluorescence and erythromycin-sensitive phenotype.

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References

- Bierne, H and Michel, B (1994). Mol Microbiol 13:17-23
- Leenhouts, KJ, Kok, J and Venema, G (1991). J Bacteriol 143:4794-4798

- Lin, M-Y, Harlander, S and Savaiano, D (1996). *Appl Microbiol Biotechnol* 45:484-489
- McIntyre, DA and Harlander, SK (1993). *Appl Microbiol Biotechnol* 40:348-355
- Mercenier, A (1990). *FEMS Microbiol Rev* 87:61-77
- Mollet, B, Knol, J, Poolman, B, Marciset, O and Delley, M (1993). *J Bacteriol* 175:4315-4324
- Prasher, DC (1995). *Trends in Genet.* 11:320-323
- Solaiman, DKY and Somkuti, GA (1993). *Plasmid* 30:67-78
- Solaiman, DKY and Somkuti, GA (1995). *J Ind Microbiol* 15:39-44
- Solaiman, DKY, Somkuti, GA and Steinberg, DH (1992). *Plasmid* 28:25-36
- Somkuti, GA and Solaiman, DKY (1997). *Curr. Microbiol.* (In Print)
- Somkuti, GA, Solaiman, DKY and Steinberg, DH (1997). *Plasmid* 37:119-127
- Somkuti, GA and Steinberg, DH (1986). *J Ind Microbiol* 1:157-163
- Somkuti, GA and Steinberg, DH (1988). *Biochimie* 70:579-585
- Yang, F, Moss, LG and Phillips, Jr., GN (1996). *Nature Biotechnol.* 14:1246-1251