Journal of Biotechnology 152 (2011) 49–53

Contents lists available at ScienceDirect

Journal of Biotechnology

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

A novel vector for lactic acid bacteria that uses a bile salt hydrolase gene as a potential food-grade selection marker

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ARTICLE INFO

Article history:
Received 22 July 2010
Received in revised form 4 January 2011
Accepted 21 January 2011
Available online 28 January 2011

Keywords:
Bile salt hydrolase
Food-grade vector
Selection marker
Lactic acid bacteria

ABSTRACT

A novel vector pM4aB for lactic acid bacterial was developed using a bile salt hydrolase gene from *Lactobacillus plantarum* as a potential food-grade selection marker. The 3.0-kb pM4aB consisted of the replicon of *Lactobacillus* plasmid pM4, a multiple cloning site and the *bsh* gene, which was constructed by elimination of a 5.5-kb non-food-grade DNA fragment from an 8.5-kb intermediate vector pBEmpM4aB. For electroporation into *Lactobacillus paracasei* X9, a high transformation efficiency of $4.0 \pm 1.0 \times 10^4$ CFU/μg plasmid DNA was yielded with 0.1% (wt/vol) glycodeoxycholic acid sodium selection. A high segregation stability of the vector was also observed as only 0.1% plasmid was lost after 50 generations of growth without selection pressure. The application potential of pM4aB was further confirmed by expression of a catalase gene from *Lactobacillus sakei* in *L. paracasei*. These results revealed that the novel vector pM4aB constructed in this study would be a useful tool for genetic modification of the industrially important LAB.

1. Introduction

Lactic acid bacteria (LAB) are widely used for the traditional and industrial manufacture of fermented food. For decades, a range of vectors equipped with antibiotic resistance markers have been constructed for genetic modification of LAB (Pouwels and Leer, 1993; de Vos, 1999). Because of the safety concern about the antibiotic resistance genes, in recent years, various potential food-grade selection markers have been developed for LAB based on their natural properties such as sugar fermentation (Boucher et al., 2002; Labrie et al., 2005), amino acid metabolism (Bron et al., 2002; Sridhar et al., 2006), nucleotide biosynthesis (Sasaki et al., 2004; Defoor et al., 2007) and bacteriocin resistance or immunity (Allison and Klaenhammer, 1996; Takala and Saris, 2002). Meanwhile, several novel selection markers have also been exploited based on environmental stress resistance such as heat shock protein, cadmium or copper resistance and bacteriophage resistance (El Demerdash et al., 2003; Liu et al., 2005; O'Sullivan et al., 2001). It is noteworthy that the resistance gene as a selection marker could simultaneously enhance the survival of the recombinant host strains under corresponding stress condition.

Bile salts play an essential role in the emulsification and solubilization of lipids. Consequently, they would exhibit antimicrobial activity by disrupting cell membranes and cellular homeostasis (Begley et al., 2005). Previous studies suggested that a common bile resistance mechanism in LAB was strongly correlated to the presence of bile salt hydrolase (BSH) activity, which probably exerts a detoxification effect by catalyzing the hydrolysis of glycine or taurine-conjugated bile salts into amino acid residues and unconjugated bile salts (De Smet et al., 1995; Grill et al., 2000; Lambert et al., 2008). A strong correlation has been observed between BSH activity and the habitat of a specific bacterial species or strain (Tanaka et al., 1999). BSH activity can usually be detected in commensal inhabitants of the gastrointestinal tract such as *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, *Clostridium* and *Bacteroides* spp. (Begley et al., 2006). Therefore, BSH possesses a great potential to be a food-grade selection marker for industrial LAB strains from habitats like milk or vegetables from which bile salts are absent. In this study, we report the development and application of a novel vector based on a high-copy-number *Lactobacillus plantarum* endogenous plasmid pM4 replicon and a *bsh* gene as a potential food-grade selection marker.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* DH5α used as a cloning host was cultured at 37 °C in Luria-Bertani (LB) medium with vigorous

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0168-1656/$ – see front matter.
doi:10.1016/j.jbiotec.2011.01.018

Table 1
Bacterial strains and plasmids used in this work.

Strains or plasmids	Relevant features	Reference or source
Strains		
E. coli DH5α	Transformation host for cloning	Transgen, Beijing, CN
L. paracasei X9	Wild-type strain, BSH negative, catalase negative	This work
L. plantarum WQ0815	Donor strain for the cloning of *bsh* gene	This work
L. sakei YSI8	Donor strain for the cloning of heme-dependent catalase gene *katA*	An et al. (2010)
Plasmids		
pBEmpM4a	Em^R, $Kana^R$, intermediate shuttle vector for *E. coli* and *Lactobacillus*	Yin et al. (2009)
pBEmpM4aB	pBEmpM4a derivative carrying *bsh* gene	This work
pM4aB	Food-grade vector, pBEmpM4aB derivative eliminating *Kpn* I non-food-grade fragment	This work
pM4aBK	pM4aB derivative carrying *katA* gene	This work

shaking. *Lactobacillus* was grown in de Man-Rogosa-Sharpe (MRS) medium at 37 °C under anaerobic conditions. For BSH activity assay, 0.1% (wt/vol) glycodeoxycholic acid sodium (GDCA; Sigma G9910, St. Louis, USA) was supplemented in MRS medium. For heme-dependent catalase activity detection, 30 μM heme was added to MRS medium. When needed, antibiotics were added at the final concentration as follows: 50 μg/ml kanamycin or 500 μg/ml erythromycin for *E. coli*; 5 μg/ml erythromycin for *Lactobacillus paracasei*.

2.2. DNA manipulation techniques

Standard DNA manipulation techniques were performed as described by Sambrook and Russell (2001). Bacterial genomic DNA was prepared using the TIANamp Bacteria DNA Kit following the manufacturer's procedures (Tiangen, Beijing, CN). Plasmid DNA from *E. coli* was isolated using the High Purity Plasmid Mini Isolation Kit according to the manufacturer's instructions (Bioteke, Beijing, CN). Plasmid DNA from *Lactobacillus* was isolated as described previously (Yin et al., 2008). Restriction endonuclease digestions were conducted according to the supplier's instructions (Takara, Dalian, CN). DNA ligation was performed using the DNA Ligation Kit according to the manufacturer's instructions (Takara, Dalian, CN). Standard heat shock transformation method was used to introduce DNA to *E. coli* DH5α (Sambrook and Russell, 2001) and electroporation was used for transformation of *L. paracasei* as previously described with some modifications (Yin et al., 2009). Briefly, 40 μl of competent cells mixed with 0.5 μg of purified DNA were transferred into an ice cold electroporation cuvette and the electric pulse was delivered using the parameter settings as follows: 1.5 kV, 400 Ω and 25 μF. 1 ml of the recovery medium (MRS supplemented with 0.3 M sucrose and 0.1 M $MgCl_2$) was immediately added after electric shock. In general, the recovery culture was incubated at 37 °C for 3 h under anaerobic conditions. However, for electroporation of the intermediate vector pBEmpM4aB with GDCA selection, the recovery time was elongated to 12 h. The results of transformation frequency corresponded to the mean of three independent parallel experiments.

2.3. Construction of an intermediate vector carrying both bsh and erythromycin resistance gene

The *bsh* gene with its native promoter was amplified by PCR from the genomic DNA of *L. plantarum* WQ0815 using a set of primers bshF (5′-AAC TGC AGG TAC CAG GTA AGG ATA AGC AGG T-3′) and bshR (5′-CGC GGA TCC ACG CGT TTA GTT AAC TGC ATA GTA TTG TG-3′). The primers were designed according to the *bsh1* gene located in the genome of *L. plantarum* WCFS1 (GenBank accession no. AL935262) (Lambert et al., 2008) and relevant restriction enzyme sites (underlined) for subsequent cloning were introduced: *Pst* I, *Kpn* I for bshF and *Bam*H I, *Mlu* I for bshR. Amplification reactions were performed using *TakaRa Ex Taq* DNA polymerase following the manufacturer's recommendations (Takara, Dalian, CN). The amplicon was digested by *Pst* I and *Bam*H I, and then inserted into the *E. coli–Lactobacillus* shuttle vector pBEmpM4a carrying a erythromycin resistance gene. The ligation mixture was transformed into *E. coli* DH5a and transformants were screened on LB agar plates containing erythromycin. The recombinant plasmid, designated as pBEmpM4aB (Fig. 1), was sequenced and further analyzed with DNAMAN software package and BLAST Program at NCBI against the GenBank database (Altschul et al., 1990).

2.4. Evaluation of bsh as a selection marker in L. paracasei

A bile salt survival assay was performed to investigate resistance of *L. paracasei* X9 to GDCA. In brief, overnight cultures were inoculated (1%) into fresh MRS broth containing a concentration range (0–0.5% [wt/vol]) of GDCA. Cell growth was measured by viable cell count.

To investigate heterologous expression of *bsh* in the BSH-deficient strain *L. paracasei* X9, the plasmid pBEmpM4aB was introduced into this host by electroporation with erythromycin selection. BSH activity was examined by a bile salt plate assay as described by Dashkevicz and Feighner (1989). Briefly, overnight cultures grown in MRS broth were streaked on MRS agar plates supplemented with 0.1% (wt/vol) GDCA. BSH activity was indicated by precipitate halos of deconjugated bile acid around active colonies. In order to further confirm the concentration of GDCA selection, pBEmpM4aB was re-transformed into *L. paracasei* X9 and transformants were screened on MRS agar plates supplemented with 0.1% (wt/vol) GDCA.

2.5. Construction of a potential food-grade vector and the segregation stability assay

The construction strategy of the vector pM4aB is shown in Fig. 1. The intermediate vector pBEmpM4aB was digested with *Kpn* I to eliminate the non-food-grade DNA fragment containing the erythromycin resistance gene, resulting in the food-grade fragment designated as pM4aB. The *Kpn* I-digested pM4aB was self-ligated with the T4 DNA Ligase (Takara, Dalian, CN) and the purified ligation mixture was then introduced into *L. paracasei* X9 by electroporation. Transformants were screened on MRS agar plates supplemented with 0.1% (wt/vol) GDCA. Plasmid profile and BSH activity assay were used to further identify the transformants. The quantitative determination of BSH activity was performed as previously described by Tanaka et al. (1999). The wild type strain of *L. paracasei* X9 was used as a negative control. The result corresponded to the mean of three parallel assays.

For segregation stability test, *L. paracasei* X9 harboring pM4aB incubated to stationary phase was consecutively inoculated to fresh MRS broth without selection pressure for 50 generations. An appropriate dilution of the suspension was then spread on one MRS agar plate with 0.1% (wt/vol) GDCA (for determination of the number of