ELSEVIER
FEMS Microbiology Letters 161 (1998) 107–114
FEMS MICROBIOLOGY LETTERS

A new theta-type thermosensitive replicon from *Lactoccocus lactis* as an integration vector for *Enterococcus faecalis*

Jacques Frère [a,*], Abdellah Benachour [b], Jean-Christophe Giard [c], Jean-Marie Laplace [a], Sigrid Flahaut [d], Yanick Auffray [a]

[a] *Laboratoire de Microbiologie de l'Environnement, IRBA, Université de Caen, F-14032 Caen cedex, France*
[b] *Institut de Biochimie, rue du Musée, 5, Université de Fribourg, CH-1700 Fribourg, Suisse*
[c] *Department of Microbiology and Immunology, University of South Alabama, MSB 2096, Mobile, AL, USA*
[d] *Laboratoire de Biologie Cellulaire et Moléculaire, Université du Littoral - Côte d'Opale, BP 299, F-62228 Calais cedex, France*

Received 26 November 1997; revised 29 January 1998; accepted 3 February 1998

Abstract

We isolated a replication thermosensitive mutant of the theta-type lactococcal pUCL22 replicon. An improved version of this thermosensitive replicon was obtained by fusioning the replication *repA* gene with the downstream *repB* gene. The resulting plasmid was named pUCB3522Ts. It is highly instable at 42°C in *Enterococcus faecalis*. Integration into the chromosome via homologous recombination was monitored using the *npr* gene of *E. faecalis* JH2-2 as a target. A 513 bp PCR amplification product from an internal region of this *npr* gene was cloned into pUCB3522Ts. Integration of this construction into the JH2-2 *npr* gene was selected by shift temperature, from 30°C to 42°C. 85% of the analysed clones showed integration into the *npr* gene, demonstrating the practicality of this thermosensitive replicon as a genetic integrative tool for *E. faecalis*. © 1998 Published by Elsevier Science B.V.

Keywords: Thermosensitive replicon; *Lactococcus lactis*; *Enterococcus faecalis*; Theta replication; Integration vector

1. Introduction

In lactic acid bacteria (LAB), genetic tools to provide integration into the chromosome, either by homologous recombination, by transposition or by bacteriophage integrase already exist [1,2]. Such systems are based upon two main delivery systems. The first one makes use of replicons which are non-functional in Gram-positive bacteria, such as pBR322 derivatives [3], or non-replicative deleted plasmids which need a specific host providing the essential replicative element encoded by its chromosome for propagation [4]. More often, these plasmids harbour genetic markers which encode antibiotic resistance. Plasmid integration into the chromosome is easily detected since the sole resistant bacteria are those carrying the resistant gene integrated into their genome. Limitation of this technique is the need of an

* Corresponding author. Tel.: +33 (2) 31 56 55 23; Fax: +33 (2) 31 56 53 11; E-mail: frere@ibba.unicaen.fr

PII S0378-1097(98)00056-1

efficient transformation procedure to select in the same step bacterial transformation and plasmid integration.

The second delivery integration system is based on thermosensitive (Ts) replicons. The general procedure in this case progresses in two steps. The first one is the establishment of the plasmid in the target strain at the permissive temperature. The second step is the cure of replicative plasmids by growing cells at non-permissive temperature without plasmid selection pressure, followed by screening integrated plasmid using the selection pressure on plates. The advantage of this procedure is that it works even when transformation efficiency is low. The two most used thermosensitive replicons derivatives from short rolling circle replicating (RCR) plasmids, namely pE194 [5], an erythromycin resistance plasmid from *Staphylococcus aureus*, and pWV01 [6], a cryptic plasmid from *Lactococcus lactis*. The corresponding thermosensitive replicons have given a widely used profuse progeny [1,7].

Some natural plasmids exhibit replicative thermosensitivity in particular contexts. The closely related pWV01 and pE194 plasmids are instable at 45°C or 51°C in *Bacillus subtilis* [7]. The lactococcal theta-type pSK11L replicon from *L. lactis* subsp. *cremoris* SK11 is thermosensitive at 39°C when introduced into some other lactococcal strains [8]. This plasmid have allowed construction of thermosensitive derivative for genomic integration, but integration occurs only in fraction of the lactococcal population and an average of 44% or 96% of the cells, depending on the strain, still contain free plasmids [9].

RCR plasmids are generally considered as poor vectors compared to theta-type because of their segregational and structural instabilities and because of the better capacity of theta-type replicon to carry and stably maintain very large cloned DNA fragments [10]. Lack of a useful theta-type thermosensitive replicon for LAB conducts us to develop such a tool using the *Rep*22 replicon [11] as a starting point. *Rep*22 from the lactose-proteinase plasmid pUCL22 of *L. lactis* subsp. *lactis* CNRZ270 is a member of a theta-type replicon family widely spread in LAB [12] which includes the conditional thermosensitive pSK11L plasmid, suggesting the feasibility of our project.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Escherichia coli XL1-Blue (Stratagene) was used for plasmid construction. *Enterococcus faecalis* JH2-2 (D. Clewell, University of Michigan) was used for thermosensitive plasmid screening, genomic integration assays and plasmid segregational analysis, as well as *L. lactis* subsp. *lactis* MG1614 [13].

pBlue-Script SK(+) and pCR-Script plasmids are supplied by Stratagene. pUCB3522 [11], a pGEM3Zf(+) (Promega) based plasmid contains the replicative region of pUCL22, the theta-type lactose protease plasmid of *L. lactis* subsp. *lactis* CNRZ270 and the erythromycin rRNA methylase (*erm*) gene of pAT110 [14]. pUCB3529 [11], pUCB3530 and pUCB3531 are pUCB3522 derivatives harbouring *Nco*I fragments of the λ phage DNA [15].

pUCB3522Hy4 is a pUCB3522 thermosensitive derivative obtained by hydroxylamine mutagenesis, and pUCB3522Ts was obtained from pUCB3522-Hy4 by removing its *Nco*I restriction site using S1 nuclease after *Nco*I digestion followed by ligation.

pUCB3522Ts-npr is a pUCB3522Ts derivative harbouring 513 bp of the *E. faecalis* JH2-2 *npr* gene obtained by PCR amplification cloned into the *Srf*I site of the pCR-Script and then transferred as a *Eco*RI/*Sal*I fragment into the *Eco*RI/*Sal*I sites of pUCB3522Ts.

E. faecalis was grown in M17 medium [16] containing 0.5% (w/v) glucose (GM17) at 37°C, or at specific temperatures as required. *E. coli* was grown at 37°C in 2TY medium [17]. Ampicillin (Am, 100 μg/ml, Sigma) for *E. coli* and erythromycin (Em, 10 μg/ml for *E. faecalis* and 300 μg/ml for *E. coli*, Sigma) were added to the media as required.

2.2. DNA manipulations and genetic transformations

Plasmid DNA was isolated from *E. coli* and *E. faecalis* cells by the alcalin lysis method [17]. RNase A (100 μg/ml) was added in the first step of plasmid DNA extraction. Restriction and modification enzymes were purchased from Boehringer-Mannheim, Amersham and Stratagene and used as recommended by the suppliers. Polymerase chain reaction

(PCR) amplification of a 513 bp fragment of the *E. faecalis npr* gene (EMBL accession number X62755 [18]) was realised using the following synthetic oligonucleotides: from the nucleotide position 1071 to 1094 of the deposited sequence, 5′-GTTATATTGGGATTGAAGCTGCCG-3′ (oligonucleotide npr-DIR), and from 1584 to 1562, 5′-AAGCCAGAAGATCCTTGAACACC-3′ (oligonucleotide npr-REV), with a Progene apparatus (Techne). PCR products were purified and DNA fragments were recovered from agarose gels using the Sephaglas BandPrep Kit of Pharmacia. PCR purified products were cloned into the *Srf*I site of pCR-Script using the pCR-Script Amp cloning kit (Stratagene). Bacteria were transformed by electroporation as previously described [19]. Other standard techniques were those of Sambrook et al. [17].

2.3. DNA sequencing

DNA was sequenced using the dideoxynucleotide chain termination method [20] with [α-^{35}S]dATP (Amersham) using the T7 Sequencing kit of Pharmacia. Cloned PCR product was sequenced after cloning into pCR-Script using M13 Reverse and M13 Universal primers (Pharmacia). The pUCL22 replication region of the pUCB3522 derivatives was sequenced using synthetic oligonucleotides. DNA sequences were analysed using the MacVector software (Kodak).

2.4. Plasmid mutagenesis

Hydroxylamine mutagenesis was performed on pUCB3522 plasmid DNA according to the instructions of Thomas [21]. Twenty μg of DNA in 40 μl of sterile water were mixed with 580 μl of a solution of sodium phosphate buffer 0.1 M, EDTA 1 mM, pH 6 and with 400 μl of hydroxylamine 1 M, EDTA 1 mM, pH 6. The mixture was then incubated at 70°C, and aliquots were removed after 0, 120, 180 and 240 min. Samples were twice dialysed for 2 h against a solution of Tris-HCl 20 mM, NaCl 20 mM, EDTA 1 mM, pH 8.4 at 4°C and DNA was precipitated with ethanol. To determine plasmid viability these DNA preparations were used to transform *E. coli* by the method of Nishimura et al. [22]. This method was preferred to the electroporation procedure since it gives a better transformation reproducibility although its level is usually lower.

2.5. Plasmid segregational stability assays

Plasmid stability was determined as previously described [23]. To determine plasmid stability during continuous exponential growth phase, an overnight culture in GM17 supplemented with erythromycin (10 μg/ml) (Em-GM17) of a strain harbouring the analysed plasmid was diluted 1:200 in Em-GM17, and grown to an optical density (OD) at 660 nm of 0.6. This culture was plated on medium with or without erythromycin at 30°C to determine the percentage of erythromycin resistant bacteria in the population, and thus the plasmid segregational stability at the onset of the experiment. One ml of the 10^{-5} dilution was used to inoculate one liter of preincubated GM17 which was grown until an OD_{660} of 0.6. The segregational stability was determined at this step, corresponding to a growth of about 25 generations without selection pressure. The culture was used to incubate a new liter of prewarmed GM17 following the same procedure.

To determine plasmid stability during discontinuous exponential growth phase, an overnight culture of a strain harbouring the analysed plasmid grown in Em-GM17 medium was diluted 1:100 in GM17 medium and allowed to grow 12 h to the stationary phase at the chosen temperature. This culture was diluted 1:100 in GM17 and allowed again to grow 12 h. This procedure was repeated and plasmid stability was determined by testing 100 colonies formed on Em-free media on media containing Em at 30°C.

2.6. Copy number determination of integrated pUCB3522Ts-npr plasmid

The copy number of the integrated form of pUCB3522Ts-npr into the *E. faecalis* JH2-2 *npr* gene was estimated on hybridised membranes by comparison of the hybridisation response of the *Eco*RI linearised plasmid and that of the *Eco*RI chromosomal fragments containing the *npr* fragment used as a probe, with the use of the Packard Cyclone Storage Phosphor System and the OptiQuant Image Analysis software (Packard instrument, USA).

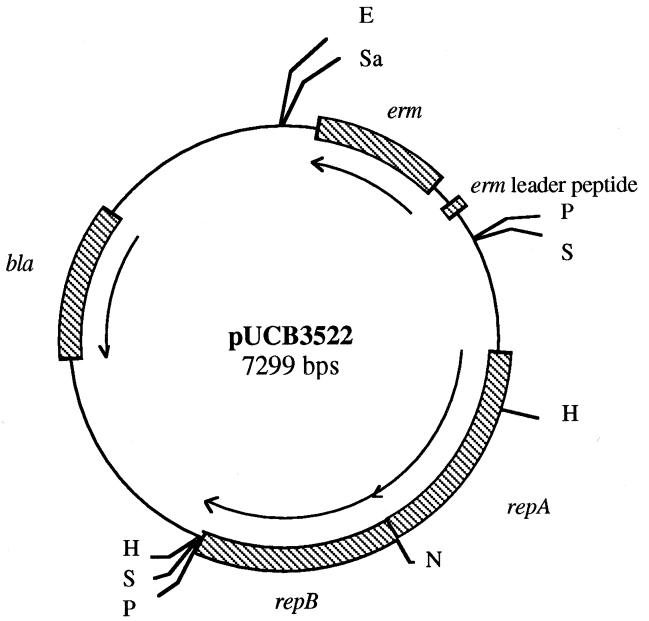

Fig. 1. pUCB3522 map. *erm*, erythromycin resistance rRNA methylase gene; *bla*, β-lactamase gene. Abbreviations for restriction enzymes are as follows: E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; P, *Pst*I; S, *Sph*I; Sa, *Sac*I.

3. Results and discussion

3.1. pUCB3522 mutagenesis

pUCB3522 (Fig. 1) harbours 2904 bp from the lactococcal plasmid pUCL22 which surrounds its replication region. It is composed by a non-coding region, the replication origin bearing DnaA box-like sequences and a stretch of direct repeats (iterons) which interacts with the replication protein RepA [11]. Following the replication origin is a coding region composed by two overlapping ORFs. The first encodes RepA, and the second, which overlaps the previous one by eight nucleotides encodes RepB for which until now no function was known in *Lactococcus*. Implication of a RepB homologous protein in the copy control of the pediococcal pUCL287 plasmid was recently demonstrated [19]. A *Nco*I site was introduced by oligonucleotide mutagenesis in the lactococcal sequence by changing one nucleotide located in the penultimate *repA* codon and the second codon of *repB* [11].

pUCB3522 DNA was mutagenised in vitro by hydroxylamine and samples obtained after various hydroxylamine length treatments were used to transform *E. coli*. Mutagenesis efficiency was estimated by the decrease of plasmid viability. After 3 h and 4 h of treatment, an average of 2% and 0.2% of the plasmid DNA still retains ability to transform *E. coli* to ampicillin resistance, respectively. These hydroxylamine treatment conditions were chosen to prepare mutagenised plasmid DNA to search for Ts plasmids in *E. faecalis* by selecting for erythromycin resistance (Em^r) at 30°C, and replica plating at 42°C. In this host, transformation efficiency of the mutated DNA preparations was from 0.6 to 0.1% compared to that of untreated DNA.

Table 1
Segregational stability of pUCB3522, pUCB3529, pUCB3530 and pUCB3531 into MG1614 during discontinuous exponential growth at 30°C

Subculture number	Plasmid-containing cells (%)	
	3	6
pUCB3522	100	86
pUCB3529	100	88
pUCB3530	18	$< 1^a$
pUCB3531	57	22

[a]No Em^r colony was detected from more than three hundred tested colonies.

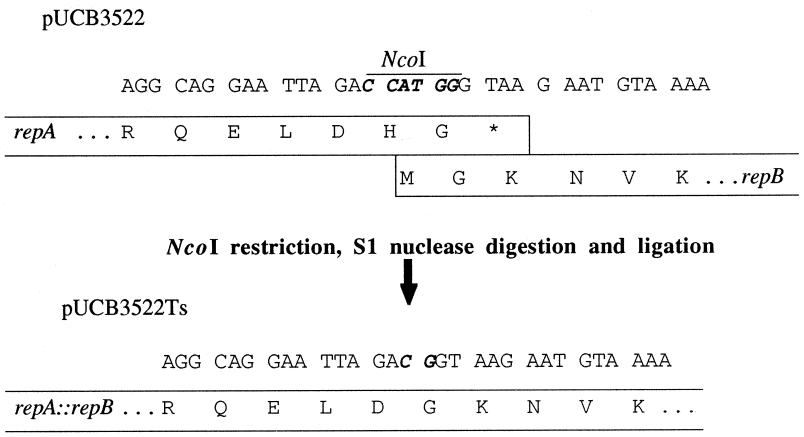

Fig. 2. Schematic representation of the overlapping nucleotide region of *repA* and *repB* genes of pUCB3522 and of pUCB3522Ts. The *Nco*I site was created by oligonucleotide mutagenesis and is not present in the wild replicon (EMBL accession number X60454).

Ten potential mutants were obtained after screening of 11 000 clones. These mutants failed to form colonies at 42°C in presence of Em. Segregational stability analysis at 30°C and 42°C of these mutants led us to determine three clusters. The first group (three clones) corresponds to mutants which have a high segregational stability, independent on the growth temperature. Since Em was added to media only during the numeration step at 30°C, and not during stability test, this first group corresponds most probably to *erm* thermosensitive mutants. Members of the second group (two clones) exhibit a thermosensitive replication, but this character was highly unstable and revertants were frequently observed. The third group consists of five members with different thermosensitivities, ranging from 80% to 30% of Em^r after 25 generations at 42°C. The most thermosensitive one, pUCB3522Hy4 was retained for further investigations. The entire lactococcal region was sequenced (data not shown). Surprisingly, the sole difference observed between pUCB3522 and pUCB3522Hy4 in this region is a nucleotide substitution in the *repA* gene resulting in the presence of a Lysin, instead of a glutamic acid residue at the codon 216 (the EMBL accession number of the wild-type is X60454). Comparison between the putative structure of the mutant protein and of the native protein does not permit us to explain the thermosensitivity of pUCB3522Hy4 (data not shown). Although we have retained the most thermosensitive mutant in our collection, its instability at the non-permissive temperature is not sufficient for construction of integration vectors.

3.2. Enhancing pUCB3522Hy4 segregational instability

Cloning of the 3967 bp *Nco*I DNA fragments of the λ phage in the *Nco*I site of pUCB3522 led to the construction of pUCB3529 [11] and in the opposite direction of pUCB3531. Cloning of the 4572 bp *Nco*I DNA fragments of the λ phage permit the construction of pUCB3530. Segregational stability analysis of these plasmids in MG1614 at 30°C is given in Table 1. Sequence analysis of these plasmids could explain their segregational stability. Into pUCB3529, the λ DNA fragment is inserted in the opposite direction of the phage coordinate (from 27868 to 23901 [15]). RepA is lengthened by four residues, -ITVS. Into

Table 2
Segregational analysis of pUCB3522Ts into JH2-2 during exponential growth phase

Generations	Plasmid-containing cells (%)	
	25	50
30°C[a]	3	0.04
37°C[a]	0.1	4.6×10^{-5}
42°C[a]	0.01	0[b]

[a]From 71% to 81% of the cells harbour the plasmid at the onset of the experiment.
[b]From 0 to 7 cells ml^{-1} harbour the plasmid into a population of 10^8 cells ml^{-1}.

pUCB3531, the λ DNA fragment is in the opposite direction, and RepA is lengthened by 19 residues (-FLVIKLSILIGCMLDADIF). In pUCB3530, the λ DNA fragment is inserted in the opposite direction of the phage coordinate (from 23901 to 19329 [15]), and RepA is lengthened by 25 residues (-KEDLKIYRTCSNAALIHILTLNYFA). The additional groups of amino acid residues in pUCB3530 and pUCB3531 RepA proteins are mainly hydrophobic. It seems that the segregational instability increases by increasing the length of the RepA protein.

Supported by these results, we tried to improve the instability of pUCB3522Hy4 by lengthening $RepA_{K216}$. For this purpose, the most convenient way

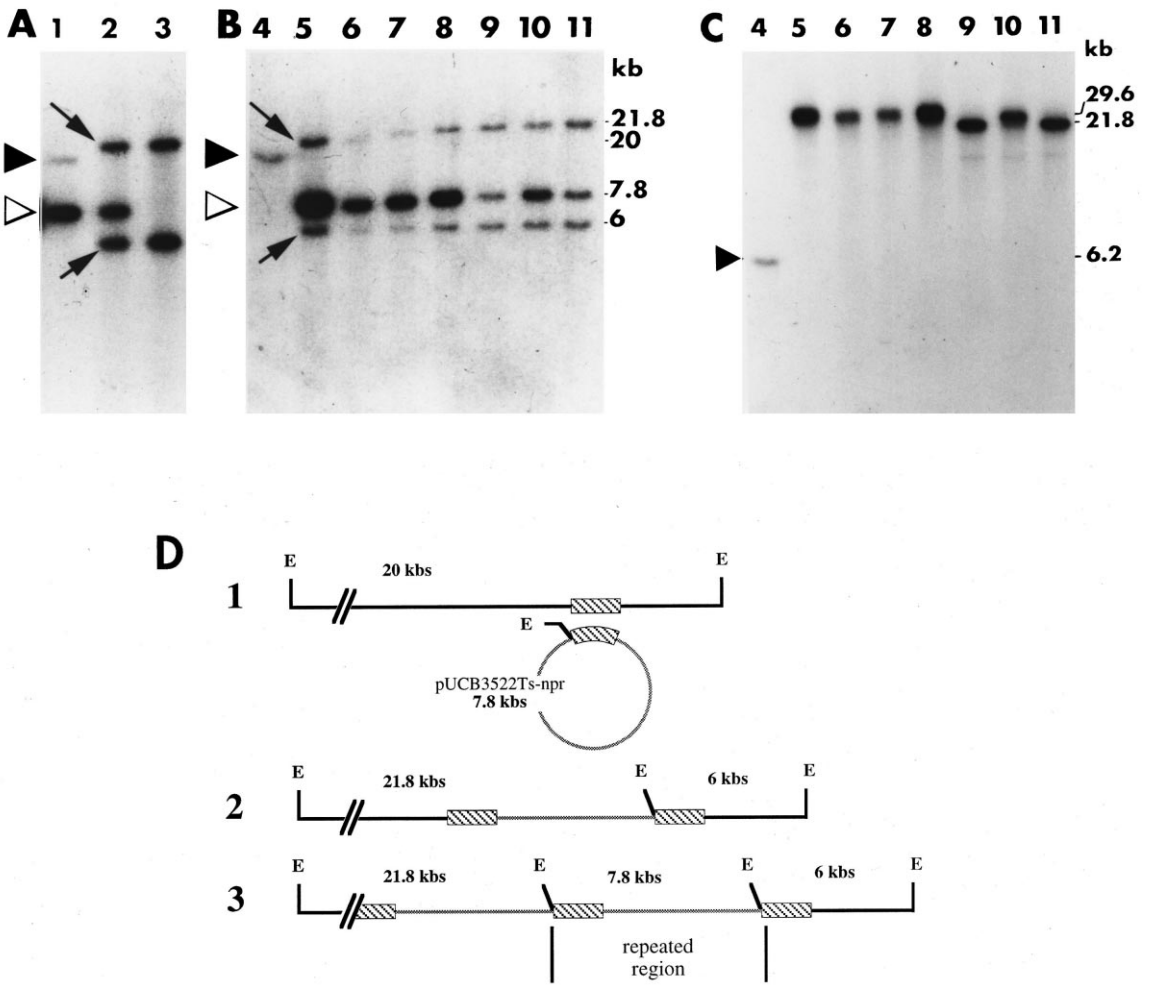

Fig. 3. A and B: Southern hybridisation analysis of *Eco*RI-digested chromosomal DNAs of JH2-2 (pUCB3522Ts-npr) clones obtained after genomic integration assay. Lanes: 1, pUCB3522Ts-npr in the autonomous state; 2 and 5–11, pUCB3522Ts-npr integrated into the *npr* gene with amplification; 3, pUCB3522Ts-npr integrated without amplification; 4, JH2-2 DNA. Arrows indicate the two fragments obtained after recombination via the *npr* gene. C: Southern hybridisation analysis of the chromosomal DNAs of panel B digested by *Cla*I. Closed and open triangles indicate the chromosome fragment bearing the *npr* gene and linearised pUCB3522Ts-npr DNA, respectively. Labeled *npr* 513 pb fragment was used as a probe. D: Schematic representation of the JH2-2 chromosome *Eco*RI fragment bearing the 513 bp *npr* fragment (hachured box) (1), and the expected structures after pUCB3522Ts-npr integration via homologous recombination (2), and amplification (3).

was to remove the *Nco*I site by treating the plasmid DNA after *Nco*I digestion by S1 nuclease, removing 4 bp and the *Nco*I site, followed by a ligation by the T4 DNA ligase. This treatment conducts to the fusion of the *repA* and *repB* genes (Fig. 2), and then to the lengthening of $RepA_{K216}$ by 118 residues. Such a clone was obtained and named pUCB3522Ts. Segregational stability assays into JH2-2 are shown in Table 2. Enumerations on GM17 and Em-GM17 of JH2-2 (pUCB3522Ts) cultures grown to OD_{660} of 0.6 on Em-GM17 broth at 30°C, 37°C and 42°C showed that elevation of growth temperature conducted to an increase of Em sensitive cells in the population of 71%, 51% and 45%, respectively (average of three enumerations) corresponding to the loss of the plasmid despite the presence of erythromycin in the growth medium.

pUCB3522Ts propagated into *E. faecalis* JH2-2 shows thermosensitive replication. Thus, this Ts replicon seems to be a good candidate for construction of chromosome integration delivery systems.

3.3. Genomic integration assays

In order to test the ability to deliver genomic integration, we have introduced into pUCB3522Ts a JH2-2 chromosomal fragment for monitoring homologous recombination. We have chosen arbitrarily the *npr* gene as an integration target. An internal gene fragment of *npr* was obtained by PCR amplification, using the synthetic nprDIR and nprREV oligonucleotides. The resulting 513 bp product was cloned into the *Srf*I site of the pCR-Script after purification and polishing. One of the resulting plasmid was chosen for the next steps. In order to check the cloned DNA, about one hundred bases of each extremity were sequenced, confirming the presence of the expected fragment of the JH2-2 *npr* gene (data not shown; EMBL accession number X62755 [18]). The *npr* gene fragment was then transferred as an *Eco*RI/*Sac*I fragment into the *Eco*RI/*Sac*I sites of pUCB3522Ts. The resulting plasmid was named pUCB3522Ts-npr.

This plasmid was introduced by electroporation into JH2-2 and propagated at 30°C. Genomic integration was conducted as follows. JH2-2 (pUCB3522Ts-npr) was inoculated into Em-GM17 and the culture was grown overnight at 30°C. The culture was then diluted and 0.1 ml of the 10^{-3} dilution was inoculated into 100 ml of GM17 and incubated at 42°C overnight. The culture was then diluted and plated on Em-GM17 plates and incubated at 42°C. After 18 h of growth, individual colonies (early colonies) appeared on plates corresponding to dilutions 10^{-2} or 10^{-3} as well as some micro-colonies (late colonies) easily distinguishable from the previous ones. Nevertheless, if plates were further incubated at room temperature or more than 24 h at 42°C, micro-colonies grew and were no longer distinguishable from the early colonies. Thirty five early colonies were inoculated in Em-GM17 and cultures were grown at 42°C. Further cultivations were conducted under the same conditions. pUCB3522Ts-npr contains one *Eco*RI site but no *Cla*I site. Total DNA was prepared, digested by *Eco*RI or *Cla*I and probed with the *npr* PRC 513 bp fragment. Results indicate that 15% of the early clones contained free copy of the plasmid (Fig. 3, lane 1), and that 85% contained integrated plasmid into the *npr* gene, sometimes with one copy of the plasmid (Fig. 3, lane 3), but most often together with an amplification (two copies of the plasmid, Fig. 3, lanes 9 and 11; three copies of the plasmid, Fig. 3, lane 10; four copies of the plasmid, Fig. 3, lane 8; five copies of the plasmid, Fig. 3, lane 5). When pUCB3522Ts-npr was in the integrated form, free plasmid was not detected (Fig. 3, panel C). Analysis of clones grown at 30°C from ten late colonies showed the presence of free pUCB3522Ts-npr plasmid (data not shown).

We demonstrate here the capacity of this new thermosensitive replicon to be used as a genetic tool in *E. faecalis*. Since it is a theta-type plasmid, and since this replicon supports maintenance of large DNA fragments [12], it is a promising tool for the introduction of large DNA fragments into the chromosome of LAB as a delivery vector.

Acknowledgments

This study was partly financed by the Agence de l'Eau Seine-Normandie. We thanks Lisa Lefrère, Marie-Jeanne Pigny, Béatrice Cheval and Annick Blandin for their technical assistance. Pr. A. Hartke is gratefully acknowledged for helpful discussions and for critically reading the manuscript.

References

[1] Biswas, I., Gruss, A., Ehrlich, D. and Maguin, E. (1993) High-efficiency gene inactivation and replacement system for gram-positive bacteria. J. Bacteriol. 175, 3628–3635.

[2] Lillehaug, D., Nes, I.F. and Birkeland, N.K. (1997) A highly efficient and stable system for site-specific integration of genes and plasmids into the phage phiLC3 attachment site (attB) of the *Lactococcus lactis* chromosome. Gene 188, 129–136.

[3] Leenhouts, K., Kok, J. and Venema, G. (1989) Campbell-like integration of heterologous plasmid DNA into the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 55, 394–400.

[4] Leenhouts, K.J., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G. and Kok, J. (1996) A general system for generating unlabelled gene replacements in bacterial chromosomes. Mol. Gen. Genet. 253, 217–224.

[5] Horinouchi, S. and Weisblumn, B. (1982) Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibodies. J. Bacteriol. 150, 804–814.

[6] Leenhouts, K., Tolner, B., Bron, S., Kok, J., Venema, G. and Seegers, J. (1991) Nucleotide sequence and characterization of the broad-host-range Lactococcal plasmid pWV01. Plasmid 26, 55–66.

[7] Yougman, P., Poth, H., Green, B., York, K., Olmedo, G. and Smith, K. (1989) Methods for genetic manipulation, cloning, and functional analysis of sprorulation genes in *Bacillus subtilis*. In: Regulation of Prokaryotic Development (Smith, I., Slepecky, R.A. and Setlow, P., Eds.), pp. 65–87. American Society for Microbiology, Washington, DC.

[8] Feirtag, J., Petzel, J., Pasalodos, E., Baldwin, K. and McKay, L.L. (1991) Thermosensitive plasmid replication, temperature-sensitive host growth, and chromosomal plasmid integration conferred by *Lactococcus lactis* subsp. *cremoris* lactose plasmids in *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 57, 539–548.

[9] Polzin, K.M. and McKay, L.L. (1992) Development of a lactococcal integration vector by using IS*981* and a temperature-sensitive lactococcal replication region. Appl. Environ. Microbiol. 58, 476–484.

[10] Jannière, L., Bruand, C. and Ehrlich, S. (1990) Structurally stable *Bacillus subtilis* cloning vectors. Gene 87, 53–61.

[11] Frère, J., Novel, M. and Novel, G. (1993) Molecular analysis of the *Lactococcus lactis* subspecies *lactis* CNRZ270 bidirectional theta replicating lactose plasmid pUCL22. Mol. Microbiol. 10, 1113–1124.

[12] Frère, J., Benachour, A., Novel, M. and Novel, G. (1993) Identification of the theta-type minimal replicon of the Lactococcus lactis subsp. lactis CNRZ270 lactose protease plasmid pUCL22. Curr. Microbiol. 27, 97–102.

[13] Gasson, M. (1983) Plasmid complements of *Streptococcus lactis* NCDO 712 and other lactic streptococci after protoplast-induced curing. J. Bacteriol. 154, 1–9.

[14] Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C. and Courvalin, P. (1990) Nucleotide sequence of the erythromycin resistance gene of the conjugative transposon Tn1545. Nucleic Acids Res. 18, 3660.

[15] Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. (1982) Nucleotide sequence of bacteriophage lambda DNA. J. Mol. Biol. 162, 729–773.

[16] Terzaghi, B.E. and Sandine, W.E. (1975) Improved medium for lactic streptococci and their bacteriophage. Appl. Microbiol. 29, 807–813.

[17] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

[18] Ross, R.P. and Claiborne, A. (1991) Cloning, sequence and overexpression of NADH peroxidase from *Streptococcus faecalis* 10C1. Structural relationship with the flavoprotein disulfide reductases. J. Mol. Biol. 221, 857–871.

[19] Benachour, A., Frère, J., Flahaut, S., Novel, G. and Auffray, Y. (1997) Molecular analysis of the replication region of the theta-replicating plasmid pUCL287 from *Tetragenococcus* (*Pediococcus*) *halophilus* ATCC33315. Mol. Gen. Genet. 255, 504–513.

[20] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

[21] Thomas, C.M. (1987) Plasmid replication. In: Plasmids: A Practical Approach (Hardy, K.G., Ed.), pp. 7–36. IRL Press, Oxford.

[22] Nishimura, A., Morita, M., Nishimura, Y. and Sugino, Y. (1990) A rapid and highly efficient method for preparation of competent *Escherichia coli* cells. Nucleic Acids Res. 18, 6169.

[23] Frère, J., Herreman, C., Boutibonnes, P., Novel, M. and Novel, G. (1995) Segregational stability and copy number of the theta-type lactococcal replicon *Rep*22 in *Lactococcus*. Current Microbiol. 29, 1–5.