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A general system for generating unlabelled gene replacements in bacterial chromosomes

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Abstract A general system is described that facilitates gene replacements such that the recombinant strains are not labelled with antibiotic resistance genes. The method is based on the conditional replication of derivatives of the lactococcal plasmid pWV01, which lacks the *repA* gene encoding the replication initiation protein. Replacement vectors can be constructed in and isolated from gram-positive and gram-negative helper strains that provide RepA *in trans*. Cointegrate formation of the integration vectors with the chromosome of the target strain is selected by antibiotic resistance. Resolution of the cointegrate structure is identified in the second step of the procedure by the loss of the *lacZ* reporter gene present in the delivery vector. The second recombination event results either in gene replacement or in restoration of the original copy of the gene. As no antibiotic resistance marker is present in the genome of the mutant the system can be used to introduce multiple mutations in one strain. A feasibility study was performed using *Lactococcus lactis* and *Bacillus subtilis* as model organisms. The results indicate that the method should be applicable to any non-essential gene in numerous bacterial species.

Key words Conditional replication · Gene replacement · Mutagenesis · *lacZ* reporter

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Introduction

The study of chromosomal genes in molecular biological research or the modification of bacterial strains with applications in the (medical) industry often requires the replacement, by homologous recombination, of a chromosomal gene by a mutated version. The simplest and most efficient method of achieving this is to exchange the target gene by a copy that is inactivated by the insertion of an antibiotic resistance gene. However, the introduction of an antibiotic resistance marker could be undesirable for various reasons. A common strategy that avoids the use of an antibiotic resistance marker in the replaced gene is a two-step procedure described by Hamilton et al. (1989). In general, in the first step homologous recombination between the delivery vector and the chromosome results in the formation of a cointegrate that is selected by positive selection. The second step is usually based on negative selection and, depending on the nature of the delivery vector, is achieved after a temperature shift or a period of non-selective growth. Resolution of the cointegrate results either in reversion to the parental chromosomal structure or in gene replacement. Bioassays or analyses of the chromosome are required to distinguish between the two possibilities. The use of temperature-sensitive (Ts) replicons is highly efficient in this strategy (Hamilton et al. 1989; Biswas et al. 1993), but Ts plasmids could have restricted host ranges or may not be thermosensitive in certain hosts. Therefore, the development of an efficient non-replicative delivery system with a wide spectrum of potential target organisms is justified.

We have previously described a conditionally replicating vector derived from the broad-host-range rolling circle-type lactococcal plasmid pWV01 (Leenhouts et al. 1991a). From this vector (pORI) the gene encoding the replication initiation protein RepA was removed. Therefore, pORI is unable to replicate in any

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant properties	Source or reference
Strain		
Escherichia coli		
NM522	*supE*, *thi*, *Δ*(*lac-proAB*), *Δhsd5*(r^-, m^-) [F′, *proAB*, *lacI*^{q}*ZΔM15*]	Stratagene (La Jolla, Calif.)
MC1000	F^-, *araD139* (*ara ABC-leu*)*7679*, *galU*, *galK*, *lacX74*, *rspL*, *thi*	Weinstock et al. (1983)
EC1000	$RepA^+$ MC1000, Km^r, carrying a single copy of the pWV01 *repA* gene in the *glgB* gene	This work
Bacillus subtilis		
BS110	$RepA^+$ DB104 (Kawamura and Doi 1984), Cm^r, carrying multiple copies of the pWV01 *repA* gene in the *add* gene region	Leenhouts, unpublished
BD393	*trpC2*, *lys*, *thyA*, *thyB*	Gryczan and Dubnau (1982)
IS233	*trpC2*, *phe*, *spoOHΔHind*	Weir et al. (1984)
QB4371	*trpC2*, *degU32*(*Hy*)::*aphA3*	Msadek et al. (1991)
Lactococcus lactis		
MG1363	Plasmid-free NCDO712	Gasson (1983)
MG1363*acmAΔ1*	Derivative of MG1363 containing a 703 bp *Sac*I-*Spe*I deletion in *acmA* gene	Buist et al. (1995)
LL108	$RepA^+$ MG1363, Cm^r, carrying multiple copies of the pWV01 *repA* gene in the random chromosomal fragment A (Leenhouts et al. 1990)	Leenhouts, unpublished
LL302	$RepA^+$ MG1363, carrying a single copy of the pWV01 *repA* gene in the *pepXP* gene	Leenhouts, unpublished
Plasmid		
pUC23rep3	Ap^r, carrying the pWV01 *repA* gene under control of promoter P23	Leenhouts et al. (1991a)
pEC1	Km^r, Tc^r, pKVB2 (Kiel et al. 1987) carrying the *repA* gene of pWV01	Law et al. (1995)
pUC19E	Ap^r, Em^r, pUC19 (Yanisch-Perron et al. 1985) carrying the Em^r of pE194 (Horinouchi and Weisblum 1982) in the *Sma*I restriction site	Laboratory collection
pTC2	Ap^r, Tc^r, pMTL25 (Chambers et al. 1988) carrying the Tc^r of pLS1 (Lacks et al. 1986) in the *Sma*I restriction site	Laboratory collection
pUK21	Km^r, *lacZα*	Vieira and Messing (1991)
pUK24	Km^r, Tc^r, pUK21 carrying the Tc^r gene of pTC2 in the *Xho*I restriction site	This work
pMG60	Em^r, pMG36e carrying the *E. coli lacZ* gene	van de Guchte et al. (1991)
pWV01	Cryptic plasmid of *L. lactis* Wg2	Leenhouts et al. (1991c)
pORI24	Tc^r, ori^+ of pWV01, replicates only in strains providing *repA in trans*	This work
pORI28	Em^r, ori^+ of pWV01, replicates only in strains providing *repA in trans*	This work
pORI240	Tc^r, $LacZ^+$, ori^+ of pWV01, replicates only in strains providing *repA in trans*	This work
pORI280	Em^r, $LacZ^+$, ori^+ of pWV01, replicates only in strains providing *repA in trans*	This work
pORI280-*pepXP*	Em^r, derivative of pORI280 specific for integrations in the *L. lactis pepXP* gene	This work
pORI280-*nrdD*	Em^r, derivative of pORI280 specific for integrations in the *L. lactis nrdD* gene	This work
pORI240-*spoOH*	Tc^r, derivative of pORI240 specific for integrations in the *B. subtilis spoOH* gene	This work
pORI280-*degU32*(*hy*)	Em^r, derivative of pORI280 specific for integrations in the *B. subtilis degU* gene	This work
pINTAA	Em^r, derivative of pORI280 specific for integrations in the *L. lactis acmA* gene	Buist et al. (1995)

bacterial strain unless RepA is provided *in trans*. The construction of integration vectors from pORI plasmids is possible by the availability of *Escherichia coli*, *Bacillus subtilis* and *Lactococcus lactis* helper strains that produce the replication protein from a chromosomally incorporated copy of *repA* (Leenhouts et al. 1991a; Law et al. 1995; Leenhouts, unpublished data). To simplify cloning procedures and to extend the application of the pORI vectors, we describe in the present paper the modular construction of pORI vectors that can be conveniently used for gene replacement strategies. The vectors carry an erythromycin (Em) or a tetracycline (Tc) resistance gene, a multiple cloning site (mcs) and the *E. coli lacZ* reporter gene. The feasibility of the system is demonstrated by the replacement of several genes in *L. lactis* and *B. subtilis*. In principle any non-essential chromosomal gene in a vast repertoire of transformable gram-positive and gram-negative bacteria can be replaced by a mutated copy using the system described here.

Materials and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. *E. coli* and *B. subtilis* were grown in TY medium (Rottlander and Trautner 1970), and *L. lactis* was grown in M17 medium (Terzaghi and Sandine 1975) containing 0.5% glucose (GM17). *L. lactis* was plated

onto GM17 agar containing 0.3 M sucrose after electrotransformation. After transformation *B. subtilis* competent cells were plated onto minimal medium (Smith et al. 1983) containing lysine, tryptophan and thymidine. Kanamycin, Em and Tc were used at final concentrations of 50, 100 and 12 μg/ml, respectively, for *E. coli*. Tc was used at a final concentration of 8 μg/ml for *B. subtilis*. Chloramphenicol and Em were used at final concentrations of 5 μg/ml for *B. subtilis* and *L. lactis*, 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-gal) was used at a final concentration of 80 μg/ml for *B. subtilis* and *L. lactis*.

Transformation

E. coli and *L. lactis* were transformed by electrotransformation as described by Zabarovsky and Winberg (1990) and Holo and Nes (1989), respectively. Competent cells of *B. subtilis* were transformed by the method of Bron and Venema (1972).

Plasmids and oligonucleotides

The plasmids used in this study are listed in Table 1. Polymerase chain reaction (PCR) primers 5′-GGA CTT GTC TGT TGA AG and 5′-ATT ATT TGA TTG GAG TT were used to analyse the MG1363::*nrdD* integrants. MG1363*nrdD*::*acmA* integrants were checked using the primers 5′-CAA GGT TAA GTC CAC G and 5′-ATT ATT TGA TTG GAG TT. The presence of the 507 bp deletion in *nrdD* or the 701 bp deletion in *acmA* in the different integrants was analysed using the PCR primers 5′-GAC TAT TTA ACG AAC TG and 5′-GGC AAG AGC ATC ATG TG. The sequences of the primers are based on sequences present in the EMBL Database under accession number X56954 (pWV01) and the GenBank accession numbers U17696 (*acmA*) and U73336 (*nrdD*).

The PCR primers used for the cloning of the mutant *spoOH* gene of *B. subtilis* IS233 were 5′-GGG AGA TCT GAG AGA GGT AGA AAC G and 5′-TTA TCT AGA TTC TCA TGC CAT TAC ACC. Primers 5′-AGA AAA GAT CTA TAT ACA ACC GAG G and 5′-GCA TCT AGA GCA CTT CAC ATT CCC GC were used to clone the *degU32*(*hy*) mutation of *B. subtilis* QB4371. The sequences of the primers are based on the sequences present in the Genbank under accession numbers M29693 (*spoOH*) and M23558 (*degU*). In both PCRs a fragment of 1 kb was generated with the mutation approximately in the middle of the fragment.

Selection for gene replacement

Transformants of *L. lactis* and *B. subtilis* in which plasmids had integrated via a single crossover were grown overnight in GM17 or minimal medium, respectively, with the appropriate antibiotics. Cultures were then diluted in medium without antibiotics to a density of 1–10 cells/ml of medium, followed by growth for approximately 30–40 generations. Dilutions of the cultures were spread onto agar plates containing X-gal. After 12–48 h white colonies were selected for further analysis.

Bioassays

PepXP-deficient mutants were identified using a PepXP plate assay as described before (Leenhouts et al. 1991b). AcmA activity was analysed as described by Buist et al. (1995). SpoOH-deficient mutants were identified as previously described (Schaeffer et al. 1965).

The *degU32*(*hy*) mutation was identified by transferring the colonies to TY agar plates containing 0.8% skimmed milk. Colonies containing the mutation produce halos on this type of agar plate, whereas wild-type colonies do not.

DNA isolation and analysis

Chromosomal DNA was isolated as previously described (Leenhouts et al. 1990). Plasmid DNA was extracted according to the method of Birnboim and Doly (1979) with the modification made by Seegers et al. (1994). Large-scale plasmid isolation was done by CsCl gradient purification (Maniatis et al. 1982). DNA transfer to Qiabrane Nylon Plus membranes (Qiagen, Düsseldorf, Germany) was according to the protocol of Chomczynski and Qasba (1984). Probes were labelled using the ECL Labelling Kit and hybridization and probe detection were carried out according to the instructions of the ECL system manufacturer (Amersham International, Amersham, UK). The PCR was carried out with super *Taq* DNA polymerase according to the instructions of the supplier (HT Biotechnology, Cambridge, UK) on chromosomal DNA.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and immunodetection

Cell extracts of overnight cultures were prepared as described by van de Guchte et al. (1990). Protein samples were subjected to SDS-PAGE according to the protocol described by Laemmli (1970) with a Protean II minigel system (Biorad Laboratories, Richmond, Calif.). Proteins separated by SDS-PAGE were transfered to BA85 nitrocellulose membrane (Schleifer & Scheull, Dassel, Germany) by the method described by Towbin et al. (1979). Endopeptidase antigen was detected with polyclonal endopeptidase antibodies (Tan et al. 1991) diluted 1:8000 and alkaline phosphatase-conjugated goat anti-rabbit antibodies (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions.

Results and discussion

Construction of pORI240 and pORI280

Derivatives of the lactococcal plasmid pWV01, the so-called pORI vectors, lack the gene encoding the replication initiation protein RepA and are, thus, unable to replicate unless RepA is provided *in trans* (Leenhouts et al. 1991a). The vectors pORI24 and pORI28, which were used as the basis for the integration vectors pORI240 and pORI280, were constructed as follows. The Tc resistance gene was isolated from plasmid pTC2 as a 1.6 kb *Bam*HI fragment and plasmid pUK21 was digested with *Xho*I. Prior to ligation of the two fragments, blunt ends were generated by Klenow enzyme treatment. The ligation resulted in pUK24, in which the Tc^r gene is flanked by two *Xho*I restriction sites. The 1.7 kb *Spe*I fragment of pUK24 carrying the Tc^r gene was treated with Klenow enzyme to create blunt ends. A 601 bp *Taq*I fragment of pWV01, which carries the plus origin of replication (Ori^+) but lacks the gene encoding the replication initiation protein (*repA*), was also treated with Klenow enzyme and both fragments were ligated, resulting in pORI24. The *Xho*I fragment of pORI24 carrying the

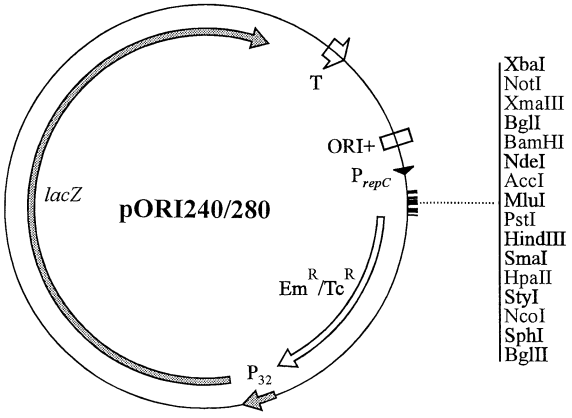

Fig. 1 Plasmid map of the Ori^+ $RepA^-$ pWV01 derivatives pORI240 (5.9 kb) and pORI280 (5.3 kb). The restriction sites indicated are unique. [Tc^r/Em^r erythromycin or tetracycline resistance gene, *lacZ* β-galactosidase gene of *E. coli* expressed under the control of lactococcal promoter P_{32} (P_{32}), *T* terminator of the lactococcal proteinase gene *prtP*, *open square* (*ORI*+) origin of replication of lactococcal plasmid pWV01, *Prepc* promoter from the *repC* gene of plasmid pWV01 (Leenhouts et al. 1991c)]

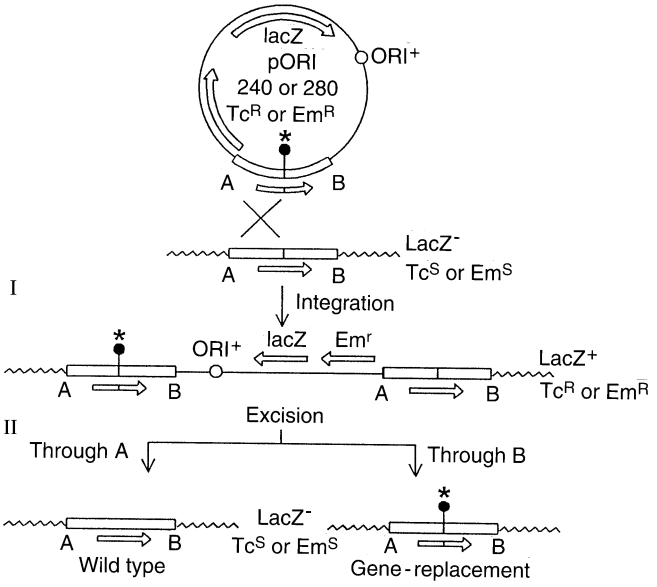

Fig. 2 Schematic representation of a two-step procedure to obtain gene-replacement recombination. The *lollipops/asterisks* indicate the presence of a mutation or a deletion or, alternatively, of a gene to be inserted in the chromosome. *A* and *B* two consecutive fragments on the plasmids and on the chromosome through which recombination could take place. In step I depicted here, only recombination via A is visualized. The end result in II would be the same if an integration had taken place through B. *Open bar* gene (fragment) of interest, *wavy line* chromosome. See Fig. 1 for a detailed description of the pORI vectors

Tc^r gene was replaced by the Em^r gene from plasmid pUC19E, located on a 1 kb *Sal*I fragment, which resulted in plasmid pORI28. Vectors like pORI24 and pORI28 have a low copy number in the *E. coli* $RepA^+$ strain EC1000, in contrast to $RepA^+$ pWV01 derivatives, which have a high copy number in *E. coli* (Kok et al. 1984).

To enable the detection of resolution of integrated plasmids in genes for which no simple detectable phenotypic difference exists between the wild-type and mutant strains, the *E. coli lacZ* reporter gene was cloned in pORI24 and pORI28. The *lacZ* expression unit from pMG60 (van de Guchte et al. 1991) was isolated as a 3.5 kb *Sal*I fragment and, after treatment of the blunt ends with Klenow enzyme, the fragment was ligated into the *Stu*I sites of pORI24 and pORI28, resulting in pORI240 and pORI280 (Fig. 1). The *lacZ* gene in these two integration plasmids is under the control of the lactococcal promoter P32 which is recognized in gram-positive and gram-negative bacteria (van der Vossen et al. 1987). Expression of *lacZ* from pORI240 and pORI280 resulted in blue colonies on X-gal plates of the $RepA^+$ strains LL108, LL302, BS110 and EC1000.

Gene replacement in *L. lactis*

We previously conducted gene replacement studies in the *pepXP* gene of *L. lactis* using a pUC derivative. Frequencies appeared to be relatively low with values as low as 10^{-6} per generation (Leenhouts et al. 1991b). Therefore, the *pepXP* region was chosen to test the pORI delivery system. Two 1.5 kb fragments of *pepXP* were cloned in pORI280. Fragment A carried the promoter and the 5′ end of the gene and fragment B the 3′ end. In the final construct, pORI280-*pepXP*, *pepXP* lacks an internal 716 bp fragment. pORI280-*pepXP* was used to transform *L. lactis* MG1363 by the two-step strategy outlined in Fig. 2. Colonies were screened for LacZ activity but not for PepXP activity during this procedure to mimic a situation in which a bioassay is lacking. We first determined through which fragment pORI280-*pepXP* had integrated. Southern blots were used to analyse 28 transformants: 19 had integrated through fragment A (MG1363::*pepXP*-A) and 9 through fragment B (MG1363::*pepXP*-B). One transformant of each type was taken and grown for 35 generations under non-selective conditions to allow resolution of the cointegrate structure. Approximately 20 000 colony forming units (4000 cfu per plate of 15 cm diameter) of each culture were plated onto agar medium containing X-gal. Five $LacZ^-$ colonies were detected among the MG1363::*pepXP*-A colonies and nine among those derived from MG1363::*pepXP*-B. All $LacZ^-$ colonies were Em^s and by Southern blot analysis (data not shown) it was shown that the numbers of gene replacements were one and three for the MG1363::*pepXP*-A and MG1363::*pepXP*-B cultures, respectively. The mutant nature of the colonies was confirmed by the PepXP plate assay. Taken together, the frequencies by which gene replacements were generated in the

Table 2 Numbers and frequencies of LacZ$^-$ colony forming units (*cfu*) and gene-replacements

Strain	Number of lacZ$^-$ cfu	Frequency of LacZ$^-$ cfu	Number of mutants	Frequency of mutants
MG1363				
::*pepXP*-A	5	7.1×10^{-6}	1	1.4×10^{-6}
::*pepXP*-B	9	1.3×10^{-5}	3	4.2×10^{-6}
::*nrdD*-A	25	2.1×10^{-4}	0[a]	$< 2.1 \times 10^{-5}$
::*nrdD*-B	59	4.6×10^{-4}	2[a]	9.1×10^{-5}
BD393				
::*spoOH*	6	2.5×10^{-5}	3	3.8×10^{-5}
BD393*spoOH*				
::*degU32(hy)*	8	1.0×10^{-4}	2	2.5×10^{-5}

Ten LacZ$^-$ cfu were tested

MG1363::*pepXP*-A and MG1363::*pepXP*-B cultures ranged between 1.4×10^{-6} and 4.2×10^{-6} per generation (Table 2). These frequencies are in agreement with our previous results (Leenhouts et al. 1991b) and demonstrate that the pORI system can be used to generate gene replacements in regions with low recombination frequencies.

To demonstrate unambiguously that with the present system genes can be replaced for which a phenotypic distinction is lacking, we chose the anaerobic ribonucleotide reductase gene *nrdD* as a target. The *nrdD* gene (2.24 kb) of *L. lactis* had not yet been described, but was identified upstream of the major peptidoglycan hydrolase gene (*acmA*) (Buist et al. 1995). Plasmid pORI280-*nrdD* carries the promoter and 5′ end of the gene in fragment A (0.8 kb) and a 3′ internal part of the gene in fragment B (1.2 kb). In this vector *nrdD* lacks an internal *Eco*RV fragment of 507 bp. After introduction of pORI280-*nrdD* into strain MG1363, 20 LacZ$^+$ Em^r cfu were analysed by PCR. Fifteen transformants (MG1363::*nrdD*-A) had integrated via fragment A, while five had integrated through fragment B (MG1363::*nrdD*-B). One transformant of each type was taken and grown non-selectively (28 generations), after which LacZ$^-$ Em^s cfu were identified among 4500 cfu of each culture (Table 2). Ten colony forming units of each type were analysed by PCR and two replacement mutants were identified originating from the MG1363::*nrdD*-B culture (9.1×10^{-5} per generation). This result was confirmed by Southern hybridization (data not shown). The fact that *nrdD* could be replaced by a mutant copy indicates that the gene is not essential under the experimental conditions applied, i.e. aerobic growth. A detailed characterization of the *nrdD* locus of *L. lactis* will be published elsewhere (G. Buist et al., manuscript in preparation).

Interestingly, frequencies of recombination between the fragments carrying the promoter and 5′ end of both *pepXP* and *nrdD* were two to three times higher in both steps of the procedure. This is in agreement with earlier observations that homologous fragments carrying a promoter show higher rates of recombination than similar-sized fragments without a promoter (Biswas et al. 1993; Buist et al. 1995).

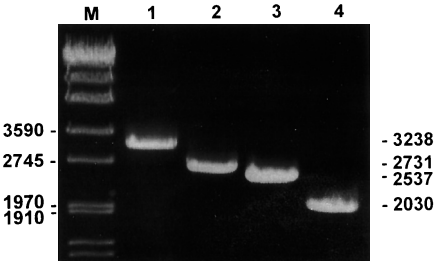

Fig. 3 Agarose (1.2%) gel electrophoresis of polymerase chain reaction (PCR) products formed on chromosomal DNAs of MG1363 (lane 1), MG1363*nrdD* (lane 2), MG1363*acmAΔ1* (lane 3), and MG1363*nrdDacmA* (lane 4). Sizes of DNA fragments (in base pairs) of bacteriophage SPPI DNA cut with *Eco*RI (molecular weight marker, lane M) and of PCR products are shown in the left and right margins, respectively

To show that the system can be used to introduce multiple mutations in one strain, pINTAA (Buist et al. 1995) was used to introduce a deletion in the *acmA* gene of MG(*nrdD*). In this case integration and excision events were not closely examined. The presence of the deletions in the genomes of the three different mutants MG1363(*nrdD*), MG1363(*acmAΔ1*), and the double mutant MG1363(*nrdD acmA*) was checked by PCR. The lengths of the PCR fragments clearly show the presence of the different deletions in the mutants (Fig. 3). These results were confirmed by Southern hybridization and, for the *acmA* mutation, by an activity assay (results not shown).

Frequently, genes are organized in operons and insertions in one gene can cause polar effects on downstream genes. This could preclude the construction of a mutation in any gene if it is followed by an essential gene under the same transcriptional control. In the pORI plasmids the *repC* promoter (P*repC*) from pWV01 (Leenhouts et al. 1991c) is present between

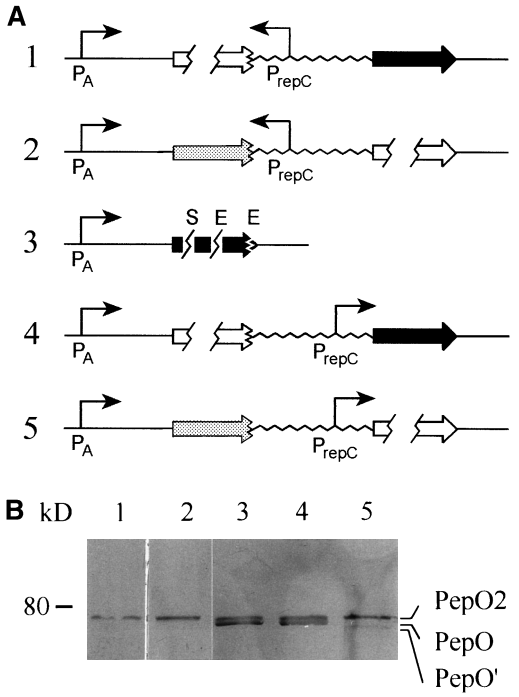

Fig. 4A Schematic representation of the chromosomal regions around the *pepO* gene of the five *L. lactis* MG1363 derivatives examined on the Western blot in **B**. The *numbers* correspond to the lane numbers in **B**. P_A and *PrepC* represent the *oppA* and *repC* promoter, respectively. For reasons of clarity, the *oppA* gene is not drawn between P_A and *pepO*. The *jagged ends* represent deletion end points. The restriction sites used to make these deletions are shown in line 3: *E Eco*RI, *S Sca*I. **B** Western blot analysis of cell-free extracts of MG1363 and four single crossover integrants in *pepO* (for details, see text). Equal amounts of protein in each lane were subjected to SDS polyacrylamide (12.5%) gel electrophoresis. The size of a molecular weight marker is indicated on the left. *PepO*, *PepO2* and *PepO′* are indicated in the right margin

Ori^+ and the mcs (Fig. 1). The lactococcal endopeptidase gene *pepO* was chosen as a chromosomal target to examine whether this promoter would be strong enough to express a gene downstream of the insertion point. *pepO* is normally transcribed from promoter P_A upstream of *oppA*, the gene immediately preceding *pepO* (Mierau et al. 1993; Tynkkynen et al. 1993; see Fig. 4A, line 3). A DNA fragment carrying *ΔpepO′*, with a truncation of the last 50 codons of *pepO* and a large deletion removing codons 37 to 285 internal to *pepO*, was cloned in two orientations behind P*repC* in pORI280. Integration of the two pORI-*pepO* constructs via sequences preceding the internal deletion results in *ΔpepO′* being transcribed from P_A (Fig. 4A, lines 1, 4). An intact copy of *pepO* is either uncoupled from P_A (Fig. 4A, line 1) or located immediately downstream of P*repC* (Fig. 4A, line 4). In the case of integration of the two pORI-*pepO* constructs via sequences behind the deletion, *pepO′* is under the control of P_A while the internally deleted form (*ΔpepO*) is either devoid of a promoter (Fig. 4A, line 2) or transcribed from P*repC* (Fig. 4A, line 5). A Western blot of cell-free extracts of the various strains is presented in Fig. 4B. The anti-PepO antibodies used also recognize a second homologous endopeptidase (PepO2), which is somewhat larger than PepO and specified elsewhere on the chromosome (Mierau et al. 1993). As is clear from Fig. 4B, lanes 4 and 5, P*repC* is active on the chromosome of *L. lactis*. The small amount of PepO′ in lane 5 is probably caused by degradation of the truncated protein. Proteolytic breakdown combined with the antisense orientation of P*repC* relative to P_A could explain the absence of the PepO′ antigen in Fig. 4B, lane 2. In conclusion, polar effects due to the insertion of the pORI vectors can be avoided by employing the transcriptional activity of P*repC*.

Gene replacement in *B. subtilis*

To demonstrate that the pORI system also operates in another gram-positive organism even under moderate transformation frequency conditions, *B. subtilis* was chosen for the consecutive introduction of the *spoOH* and *degU32*(*hy*) mutations into the same strain. The mutated *spoOH* gene was cloned as a 1 kb fragment by PCR from strain IS233 into pORI240, with the mutation approximately in the middle of the PCR fragment. The resulting plasmid pORI240*spoOH* was used to transform *B. subtilis* BD393. The route of integration was not investigated in this case. A Tc^r $LacZ^+$ transformant was taken and grown non-selectively for 40 generations before plating. Among 2000 cfu, 6 $LacZ^-$ colonies appeared, 3 of which were found to be Spo^- in a sporulation assay (3.8×10^{-5} per generation) (Table 2).

The *degU* gene from strain QB371, carrying the *degU32*(*hy*) mutation, was cloned as a 1 kb fragment by PCR in pORI280. Strain BD393::*spoOH* was transformed with the resulting plasmid pORI280*degU32*. An Em^s $LacZ^+$ colony was taken and grown non-selectively for 40 generations. Eight $LacZ^-$ colonies were identified among 2000 cfu generated from this culture (Table 2). Two of the $LacZ^-$ colonies had retained the *degU32*(*hy*) mutation (2.5×10^{-5} per generation) as judged by a halo assay using agar plates containing skimmed milk.

Comments on the method

The vectors used in this study lack their cognate *rep* gene. Thus, this type of integration vector does not rely on replication in the target host and does not depend on temperature sensitivity of a replication protein in that host. Apart from the functionality of the pORI vectors in *L. lactis* and *B. subtilis*, these vectors have been used in *E. coli*, albeit for single crossover integrations (J. Müller, unpublished data). It is conceivable

that the vectors can be used in practically any bacterial host. An exception may be some lactococcal strains harbouring pWV01-like plasmids, which could provide RepA *in trans*. A plasmid-curing step prior to the use of the pORI vectors will be necessary. Adjustment of the vectors for use in some bacterial species may be required, such as insertion of another selectable marker and replacement (of the promoter) of the reporter gene. This can easily be achieved by virtue of the modular design of vectors pORI24 and pORI28.

The pORI plasmids have the advantage over other non-replicative vectors that they allow cloning of the target gene (fragment) in gram-positive or gram-negative backgrounds. This may minimise cloning problems. If such problems should persist, construction of a homologous $RepA^+$ background may be considered. The availability of a *repA* expression cassette is convenient for this purpose.

A drawback of the method is that transformation frequencies are required that are sufficiently high to obtain at least a few integrants. Therefore, it may be necessary to optimise transformation protocols for poorly transformable strains.

In the present system the presence or absence of an expressed *lacZ* gene enabled a simple blue/white screening of colonies to visualize recombination events in the second step of the procedure. However, this system is still based on negative selection, which may result in extensive screening of colonies if recombination frequencies in the target gene are very low ($<10^{-6}$ per generation). As already indicated above, the reporter gene may be replaced by a gene that allows positive selection, if available. Nevertheless, the pORI vectors described here were found to be highly efficient. In addition to the mutations described, the pORI system has been used to mutate the *dtpT*, *pepO*, *pepT* and *acmA* genes of *L. lactis* (Mierau et al. 1993, 1994; Hagting et al. 1994; Buist et al. 1995).

The fact that no antibiotic resistance marker is left on the chromosome of a mutated strain makes the strain not only more desirable for applied purposes but also leaves the possibility of introducing more desired mutations (or genes).

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