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A lactococcal pWV01-based integration toolbox for bacteria

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Abstract. A conditionally replicating lactococcal vector system is described, based on pWV01, that is used for chromosomal integration in *Lactococcus lactis*. The system consists of plasmids that are all based on the broad-host-range lactococcal replicon pWV01 which has been deprived of its gene encoding the replication initiation protein RepA. These so-called pORI plasmids can only replicate if RepA is provided *in trans*. Special *Escherichia coli*, *Bacillus subtilis* and *L. lactis* helper strains, producing RepA *in trans* ($RepA^+$), are used as intermediate hosts for the construction of pORI integration plasmids. The presence of a lactococcal chromosomal DNA fragment in a pORI plasmid enables its chromosomal integration by homologous recombination in an *L. lactis* strain that does not produce RepA ($RepA^-$). A set of special purpose pORI vectors are available: (i) pORI280, designed to mutate or delete genes or to insert new genes in the chromosome in such a way that no heterologous DNA or antibiotic resistance markers are left in the recombinant strain, here designated as silent gene replacement; (ii) pORI19, suitable for random mutagenesis of the chromosome, and (iii) pORI13, constructed to make random single copy transcriptional fusions in the chromosome to search for (environmentally regulated) promoters. Some of these vectors have also been succesfully applied for integration in *E. coli* and *B. subtilis*. We strongly believe that the systems described here can be used in various other bacterial species.

Key words: Conditional replication, Gene replacement, Integration, Mutagenesis, Transcriptional fusions

1. Introduction

The development of tools for the analysis and modification of bacterial chromosomal genes and their expression signals has resulted in a large number of integration vectors and strategies. The essential parts of the most versatile and frequently employed vectors are usually derived from transposable elements, plasmids or combinations thereof. The integration event either relies on *rec*-independent transposition or on *rec*-dependent homologous recombination. Transposition has been a valuable tool for bacteria like *Escherichia coli* and *Bacillus subtilis* [1, 31]. However, transposable elements often have a limited host range or may have one or more transposition hot spots. In *Lactococcus lactis*, a Gram-positive mesophilic lactic acid bacterium and subject of our studies, this problem has also been encountered. Heterologous conjugative transposons like Tn*916* and Tn*919* have been used to some extent in *L. lactis*, but their use has been restricted either because transposition was site-specific or high-efficiency conjugal transfer systems were required [5, 22]. Derivatives of the *Enterococcus faecalis* transposon Tn*917* were successfully used in *L. lactis* to obtain random transposition using pLTV1 or pTV32, both of which contain a temperature-sensitive replicon of pE194 [31]. In e.g., *B. subtilis*, the temperature-sensitive property of this type of plasmid was used to establish a culture of cells containing the replicative form of the plasmid at 37 °C and to cure the cells of the plasmid at 42 °C, thereby selecting for transposition events. Since the maximum growth temperature for *L. lactis* is about 37.5 °C, a similar temperature upshift can not be used in this organism. Nevertheless, efficient transposition was obtained and cells were cured for plasmids at 30 °C by shifting a culture from medium with one antibiotic, chloramphenicol, to medium containing another antibiotic, erythromycin. The molecular basis for this phenomenon has yet to be resolved. Nevertheless, the process was efficient enough to establish a collection of environmentally regulated chromosomal promoters [7, 8].

Lactococcal transposable elements that have been used succesfully in the analysis of the *L. lactis* chromosome belong to the IS*S1* family of insertion sequences [19, 21, 23, 25]. Replicative transposition of an IS*S1* element present on a plasmid results in the integration of the entire plasmid in the chromosome, flanked by two copies of the IS element. This system has been used in combination with an *E. coli* replicon for insertional mutagenesis purposes [3, 24] and for the construction of physical and genetic maps of lactococcal chromosomes [11, 12]. A more versatile IS*S1* vector, pGh:IS*S1*, has recently been

constructed by making use of a thermosensitive derivative of the lactococcal vector pWV01. This vector replicates in the target organism at 28 °C but is lost at 37.5 °C. The use of a thermosensitive replicon uncouples transformation and transposition events which results in an important improvement of transposition frequencies. High-frequency random transposition (> 0.5%), allowing for efficient random gene inactivation, has been demonstrated with pGh:IS*S1* in *L. lactis*, *Streptococcus thermophilus* and *E. faecalis* [19]. The wide host range of pWV01-based replicons, which replicate both in Gram-negative and Gram-positive bacteria [9], offers the possibility to use this tool in a great variety of bacteria. Its successful application will depend on the thermosensitivity of the pWV01 replicon and the transposition activity of the IS*S1* element in the target organism.

The *rec*-dependent plasmid integration strategies rely on the presence in the vector of one or more DNA fragments with homology to the chromosome of the target organism. In principle any heterologous plasmid which is unable to replicate in the bacterium of interest can be used for this purpose. This approach has also been successfully taken for *L. lactis* to obtain single and double cross-over integrations [17]. However, the nature of several (potential) applications of chromosomally modified lactococcal strains demanded the development of more sophisticated integration vectors, allowing to do the necessary steps of vector construction in a homologous strain. The broad host-range lactococcal plasmid pWV01 proved to be an excellent substrate for the construction of such versatile integration vectors. Two conditionally replicating vector systems have been developed: (i) the above mentioned temperature sensitive (Ts) pWV01 derivative pG$^+$host, which was developed in the laboratory of D. Ehrlich,

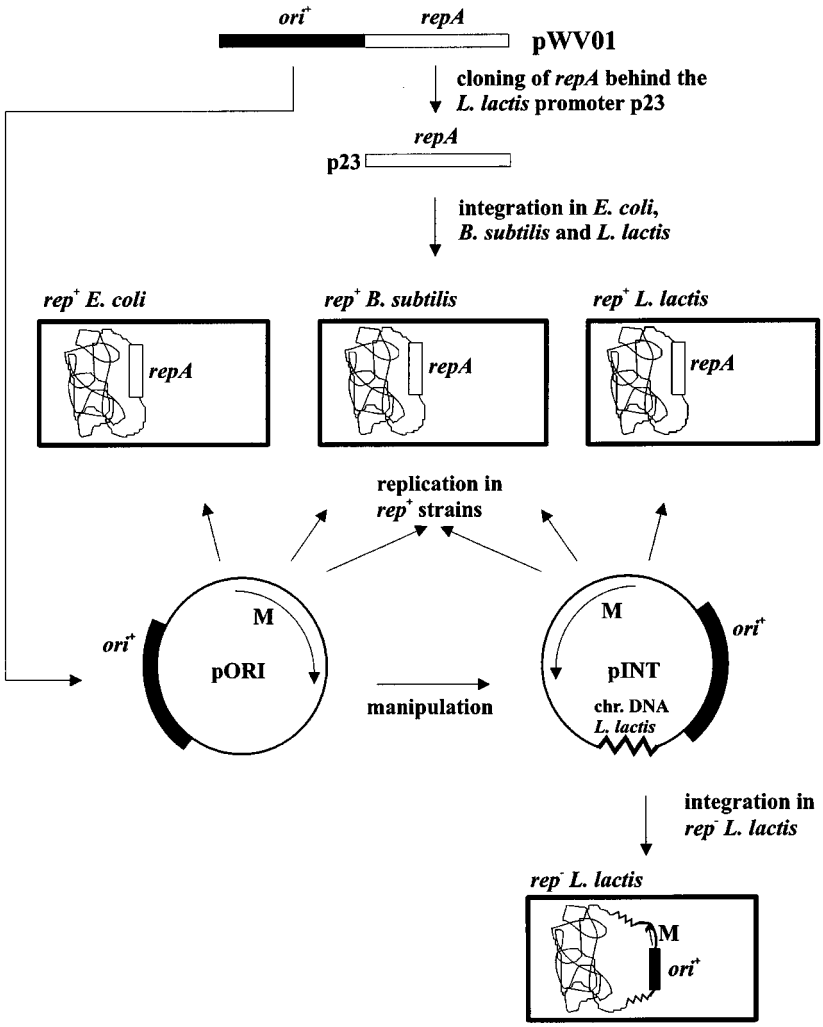

Figure 1. Schematic representation of the Ori$^+$-system. Ori$^+$ (black box): fragment containing the plus origin of replication of pWV01; RepA (white box): fragment encoding the replication initiation protein of pWV01; rep$^+$: RepA-producing strain; p23: constitutive lactococcal promoter, also active in *E. coli* and *B. subtilis*; pORI: Ori$^+$-vector that does not contain lactococcal chromosomal DNA; pINT: Ori$^+$-vector containing lactococcal chromosomal DNA; rep$^-$: strain that does not produce RepA; chr. DNA (wavy lines): chromosomal DNA; M: selectable marker.

Jouy en Josas, France [18]. The Ts-system has the important advantage that it uncouples the transformation and integration events, which bypasses the need for high transformation frequencies in the target organism. Nevertheless, in some applications it may have the disadvantage that the mutant strains have to be kept at temperatures above 37.5 °C. For more details on the Ts-system the reader is referred to the original literature [2, 18, 19]; (ii) a system in which pWV01-derivatives deprived of *repA*, the gene essential for plasmid replication, are multiplied in strains which provide RepA *in trans* and are integrated in strains which lack the *repA* gene of pWV01[Ori^+-system; 15, 17]. Figure 1 summarises the characteristics of the Ori^+-system. The pWV01 *repA* gene under the control of the lactococcal promoter P_{23}, which is expressed in Gram-positive as well as in Gram-negative bacteria [30], was integrated into the chromosomes of strains of *E. coli*, *B. subtilis* and *L. lactis* [10, 13–15]. These $RepA^+$ strains produce the RepA protein *in trans*, thus sustaining the replication of pWV01-derivatives lacking *repA*, but still carrying the recognition site for the initiation of replication (pORI plasmids). The Rep^+ helper strains are used for the construction of pORI-derivatives carrying chromosomal DNA of the target strain (pINT plasmids). Such pINT vectors fail to replicate in strains without a functional *repA* (Rep^- strains) and can thus be used to direct integration of cloned DNA. The Ori^+-system has the advantage that it operates independently from temperature shifts. However, in some applications this system may suffer from the disadvantage that relatively high transformation frequencies are required.

The pORI-vectors and strategies tailored for generating silent gene replacements, random chromosomal mutants and random chromosomal transcriptional fusions will be discussed here. A combined strategy will also be addressed, in which the Ts-plasmid is used as a helper plasmid in the Ori^+-system, to benefit from the advantages of both systems. We strongly believe that the nature of the conditionally replicating vectors described here implies that they can be successfully used in various other species of bacteria.

2. Materials

A. Equipment

1. Balances no. 1409 and BA160P.[16]
2. Gene pulser no. 165-2077 and Pulse controller no. 165-2098.[4]
3. Incubator (Heraeus) no. B5060E.[13]
4. Microcentrifuge no. 5417C.[7]
5. pH meter (Hanna Instruments) no. 8520.[13]
6. Pressure cooker canner (Presto) no. VL511080.[13]
7. Research pipettes no. 3110.[7]
8. Table top centrifuge no. 5810R.[7]
9. Vacuum dryer SpeedVac SC110-240 and Refrigerated Vapor Trap RVT100.[17]
10. Vortex IKA-Vibrofix VF1 Electronic no. 7102775.[14]

B. Supplies

1. Centrifuge tubes 50 ml no. 227.261.[9]
2. Electroporation cuvettes no. 165-2086.[4]
3. Microcentrifuge tubes, safe-lock (1.5 ml and 2 ml) no. 0030.120.086 and 0030.120.094.[2]
4. Pasteur capillary pipettes (W.U. Mainz) no. 222001.[14]
5. Petri dishes no. 631102.[9]
6. pORI13 + EC 1000.[11]
7. pORI19 + EC 101.[11]
8. pORI280.[11]
9. pVE6007.[12]
10. Research pipette tips 100 µl no. 0030.036.000.[7]
11. Research pipette tips 1 ml no. 808518.[14]

C. Media and chemicals

1. Acetic acid, glacial no. 63.[13]
2. Agar no. 4311849.[2]
3. $CaCl_2$ no. 2382.[13]
4. Chloramphenicol no. C-0378.[18]
5. Chloroform no. A3505E.[10]
6. Dimethylformamide no. 822275[13]
7. Double distilled H_2O (ddH_2O).
8. EDTA no. 2802145.[3]
9. Erythromycin E-6376.[18]
10. Ethanol no. 983.[13]
11. Glucose no. 8342.[13]
12. Glycerol no. 4094.[13]
13. Glycine no. 190.[13]
14. HCl no. 317.[13]
15. Isoamylalcohol no. 999.[13]
16. Lysozyme no. 5281.[13]
17. M17 no. 1856-17.[6]
18. $MgCl^2$ no. 5832.[13]
19. NaAc no. 6268.[13]
20. NaCl no. 6404.[13]
21. NaOH no. 6498.[13]
22. Phenol no. 206.[13]
23. Proteinase K no. 24568.[13]
24. RNase no. 109.193.[5]
25. SDS no. 1667.289.[5]
26. Sucrose no. 17714-0010.[1]
27. Tris no. 708976.[5]
28. Xgal no. OP-0020-10.[8]

3. Procedures

Some of the procedures for the pORI-system involve cloning steps in *E. coli* and general DNA techniques which are described in standard handbooks [26]. Here we describe only the *L. lactis*-specific procedures.

Silent gene replacements. One of the procedures which avoids the use of an antibiotic resistance marker to mutate a gene of interest is a two-step procedure described by Hamilton et al. [4]. In general, in the first step of homologous recombination between the delivery vector and the chromosome a cointegrate is formed which is selected for by positive selection. The second step is usually based on negative selection and, depending on the nature of the delivery vector, consists of 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. We developed a vector, pORI280 (Figure 2), which was designed for use in such a strategy (Figure 3).

A. Preparation of media and solutions

1. GM17 growth medium: dissolve 3.7 g M17 in 100 ml ddH_2O, sterilize by heat treatment using a pressure cooker or autoclave (15 min 120 °C) and store at room temperature in the dark. Add 2.5 ml 20% glucose solution just prior to use.
2. $GM17E^5$ growth medium: as in 1, add 50 µl of an erythromycin solution (10 mg/ml) before use.
3. GM17 and $GM17E^5$ agar medium: as in 1 and 2 but add 1.5 g agar before sterilization.
4. GM17 Xgal and $GM17E^5$Xgal agar medium: as in 3 but add 200 µl 4% Xgal before use.
5. GSM17gly growth medium: dissolve 3.7 g M17, 17.1 g sucrose and 1.9 g glycine in 80 ml ddH_2O, adjust the volume to 100 ml, sterilize as before and store at room temperature in the dark. Add 2.5 ml 20% glucose just prior to use. Note: the optimal amount of glycine in the medium differs among lactococcal strains e.g. for MG1363 it is 1.9 g per 100 ml while for IL1403 its 1 g per 100 ml.
6. GSM17MCery recovery medium: dissolve 3.7 g M17 and 17.1 g sucrose in 80 ml ddH_2O, adjust the volume to 100 ml, sterilize and store as before. Add 2.5 ml 20% glucose, 2 ml 1 M $MgCl_2$, 200 µl 1 M $CaCl_2$ and 500 µl erythromycin (10 µg/ml) just prior to use. Note: the 50 ng/ml erythromycin in this medium is required for induction of the expression of the erythromycin resistance gene. If selection for another antibiotic is used, erythromycin should be omitted.
7. $GSM17E^5$ agar medium: as in 3 but add 10 g

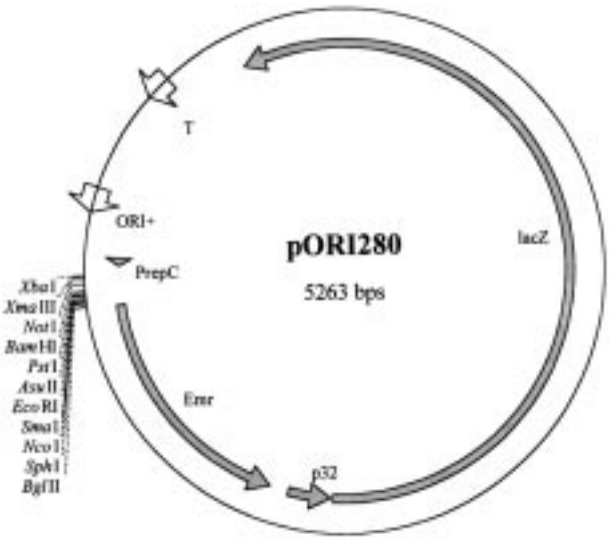

Figure 2. Plasmid map of pORI280 (5.3 kb). The restriction enzyme sites indicated are unique. Em^r, erythromycin resistance gene; *lacZ*, β-galactosidase gene of *E. coli* expressed under control of lactococcal promoter P_{32} (p32); T (open arrow), terminator of the lactococcal proteinase gene *prtP*; open arrow (ORI+), origin of replication of lactococcal plasmid pWV01; PrepC, promoter of the *repC* gene of plasmid pWV01 [16].

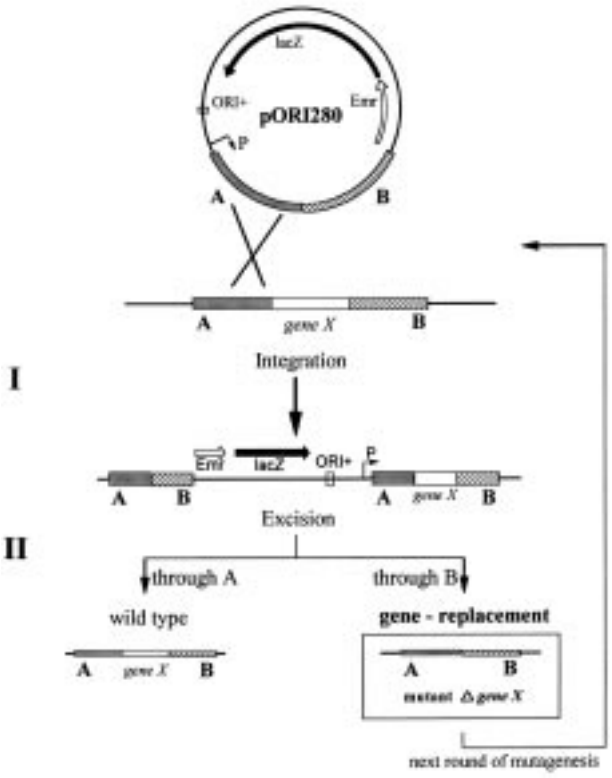

Figure 3. Scheme of a two-step procedure to obtain gene-replacement recombination. A (grey box) and B (hatched box): two fragments flanking the *geneX* to be deleted from the chromosome and through which recombination can take place. In step I depicted here, only recombination via A is visualized. The end result in II would be the same if an integration would take place through B. P: promoter P_{repC} of pWV01; ORI+ (open box): origin of replication of pWV01; lacZ (black arrow): β-galactosidase gene of *E. coli* expressed under control of lactococcal promoter P_{32}; Emr (open arrow): erythromycin resistance gene; *gene X*: target gene; Δ: deletion.

sucrose before sterilization to 80 ml ddH_2O and adjust after dissolving to 100 ml.

8. 20% glucose: dissolve 20 g glucose in 80 ml ddH_2O, adjust the volume to 100 ml and sterilize as before. Store at room temperature.
9. Erythromycin (10 mg/ml): dissolve 1 g erythromycin in 100 ml ethanol. Store at –20 °C.
10. Erythromycin (10 µg/ml): dissolve 1 mg erythromycin in 100 ml ethanol. Store at –20 °C.
11. X-gal (4%): dissolve 400 mg in 10 ml dimethylformamide and store at –20 °C.
12. 1 M $MgCl_2$: dissolve 20.3 g $MgCl_2$ in 100 ml ddH_2O and sterilize as before. Store at room temperature.
13. 1 M $CaCl_2$: dissolve 16.8 g $CaCl_2$ in 100 ml ddH_2O and autoclave as before. Store at room temperature.
14. 0.5 M sucrose/10% glycerol: dissolve 85.5 g sucrose and 57.5 ml glycerol (87%) in 400 ml ddH_2O, adjust volume to 500 ml and autoclave as before. Store at 4 °C.
15. 10 N NaOH: dissolve 40 g NaOH in 90 ml ddH_2O. Adjust the volume to 100 ml and store at room temperature.
16. 0.5 M EDTA: resuspend 14.6 g EDTA in 80 ml ddH_2O. Slowly add, while stirring, 10 N NaOH until solution becomes clear and reaches 8. Adjust volume to 100 ml and store at room temperature.
17. 1 M Tris pH 8: dissolve 12.1 g Tris in 80 ml ddH_2O. Slowly add, while stirring, concentrated hydrochloric acid untill the pH reaches 8. Adjust volume to 100 ml and store at room temperature.
18. Lysis solution: dissolve 20 g sucrose and 300 mg NaCl in 80 ml ddH_2O, add 1 ml 1 M Tris (pH8) and 2 ml 0.5 M EDTA. Adjust volume to 100 ml and autoclave as before. Store at 4 °C.
19. 10% SDS: dissolve 10 g SDS in 90 ml ddH_2O. Adjust volume to 100 ml and store at room temperature.
20. Proteinase K (20 mg/ml): dissolve 20 mg proteinase K in 1 ml ddH_2O. Store at –20 °C.
21. Chl/IAA: mix 96 ml chloroform with 4 ml isoamylalcohol. Store at room temperature.
22. 3 M NaAc pH 5.2: dissolve 24.6 g NaAc in 80 ml ddH_2O. Add, while stirring, acetic acid until the pH reaches 5.2.
23. TE: add 1 ml of Tris (pH 8) and 200 µl of 0.5 M EDTA to 99 ml ddH_2O.
24. RNase (10 mg/ml): dissolve 10 mg RNAse in 1 ml ddH_2O and incubate the sample at 100 °C for 10 min to inactivate DNase activity.

B. Preparation of cells for electrotransformation of strain MG1363 according to the method of Holo and Nes [6]

1. Inoculate strain in 5 ml GSM17gly and grow overnight at 30 °C (standing culture).
2. Add the 5 ml of overnight culture to 200 ml of fresh GSM17gly medium and grow at 30 °C to an OD_{600} of 0.4.
3. Pellet the cells by centrifugation at 4 °C in a cooled table top centrifuge using sterile 50 ml tubes (5,000 rpm, 5 min). Keep the cells on ice in between all further steps.
4. Wash the cells three times with 50 ml ice-cold 0.5 M sucrose/10% glycerol. Pellet the cells at 4 °C at 6,000 rpm for 10 min. Note: during the washing steps the cells pellet less well and higher centrifugation speeds and/or longer centrifugation times may be required to pellet all cells.
5. Resuspend the cells in 1 ml 0.5 M sucrose/10% glycerol and transfer the mixture to a 2 ml microcentrifuge tube.
6. Pellet the cells at 4 °C in a cooled microcentrifuge at 12,000 rpm for 3 min.
7. Resuspend the cells to a total volume of 1 ml using 0.5 M sucrose/10% glycerol.
8. Store aliquots of 50 µl at -80 °C.

C. Electroporation

1. Chill a 2 mm electroporation cuvette and the plasmid DNA preparation on ice.
2. Thaw electro-competent cells on ice; use an aliquot of 50 µl per transformation.
3. Prepare one sterile 2 ml microcentrifuge tube with 1 ml GSM17MCery per transformation.
4. Mix the cells with the plasmid DNA and transfer the mixture to the chilled cuvette.
5. Expose the cells immediately to a single electrical pulse (2.5 kV, 25 µF, 200 Ω).
6. Mix the cells immediately after the discharge with the GSM17MCery as follows: transfer the medium into the cuvette using a Pasteur pipette, mix and transfer the mixture back into the microcentrifuge tube.
7. Incubate the cells at 30 °C for 2 h.
8. Plate 100 µl aliquots per $GSM17E^5$ agar plate. If necessary, plate dilutions (made in GSM17MCery).
9. Incubate plates at 30 °C.

D. Gene replacement in MG1363 using pORI280 [14]

1. Insert two flanking fragments of the region to be deleted in the multiple cloning site (mcs) of pORI280 and transform to *E. coli* EC1000 ($RepA^+$). The sizes of the flanking fragments may range from 500 to 2,500 bp. It is recommended to use similar sized fragments.
2. Isolate a correct plasmid from EC1000 and transform *L. lactis* MG1363 ($RepA^-$) by electrotransformation with 0.1 to 1 µg of the pORI280 construct in a volume of 1 to 3 µl TE (step I in Figure 3).
3. Select the transformants on $GSM17E^5$ agar

plates. Colonies should be visible after 48 h of incubation at 30 °C.

4. Transfer a number of the colonies to GM17E[5]Xgal plates. The colonies should stain blue after overnight incubation at 30 °C.
5. Inoculate a number of blue colonies in 2 ml GM17E[5] each for the isolation of chromosomal DNA. Use standard Southern hybridization or PCR techniques to check whether the plasmid has integrated at the expected chromosomal location. Note: steps 3 to 5 are optional. However, we recommend to go through these steps in order to get a better understanding of the process.
6. Grow one (or more) of the integrants overnight in 2 ml GM17E[5]. Note: at this stage it may be advantageous to use those integrants which were created by integration of pORI280 through the chromosomal fragment that demonstrated the lowest recombination frequency of the two fragments present in the vector (A and B in Figure 3).
7. Dilute the overnight culture 10^6 times. A lactococcal overnight culture in GM17 medium contains about 2×10^9 cells. By inoculating 100 µl of the 10^6-fold diluted culture in 100 ml GM17 (without erythromycin), a cell density of 1 to 10 cells per ml is obtained.
8. Grow the diluted culture overnight at 30 °C. In this way nonselective growth of approximately 35 generations has been achieved. Note: overnight growth at 37 °C at this stage may enhance the excision-frequency of the integrated plasmid up to 10-fold (step II in Figure 3).
9. Plate dilutions of the overnight culture on GM17Xgal plates and incubate at 30 °C. Ideally not more than approximately 500 colonies should appear per plate (∅ 9 cm). Alternatively ∅ 15 cm plates can be used which should contain not more than approximately 2000 colonies. Ten ∅ 9 cm plates are usually enough to identify a sufficient number of white colonies.
10. Growth for 24 to 48 h on these plates should be sufficient to distinguish white from blue colonies.
11. Select white colonies and grow each of them overnight in 2 ml GM17 at 30 °C for chromosomal DNA isolation. Southern hybridization or PCR strategies should be used to distinguish between wild-type colonies and strains in which gene replacement has occurred. Alternatively, white colonies can be grown for bioassays if these allow distinction between the two types of strains.

E. Chromosomal DNA isolation

1. Inoculate strain in 2 ml GM17 and grow overnight at 30 °C (standing culture).
2. Transfer 1 ml overnight culture to a 2 ml microcentifuge tube and pellet the cells using a microcentrifuge (14,000 rpm, 3 min).
3. Wash the cells with 1 ml ddH_2O and pellet as before.
4. Resuspend the cells in 500 µl lysis solution to which lysozyme has been added (end concentration 5 mg/ml).
5. Incubate 15 min at 50 °C.
6. Add 25 µl proteinase K and mix by inversion.
7. Add 25 µl 10% SDS and mix by inversion.
8. Incubate for 1 h at 60 °C. The solution should now be completely clear.
9. Add 250 µl phenol and mix well by inversion.
10. Add 250 µl Chl/IAA and mix well by inversion.
11. Separate the two phases by centrifugation (14,000 rpm, 4 min).
12. Carefully transfer 450 µl of the aqueous (DNA-containing) upper phase to a clean 2 ml microcentrifuge tube. Do not take any of the inter- and/or phenol phase.
13. Add 100 µl ddH_2O to the DNA solution and mix by inversion.
14. Repeat steps 9 to 12.
15. Add 45 µl 3 M NaAc pH 5.2 to the DNA solution and mix by inversion.
16. Add 1 ml ice cold 96% ethanol and mix by inversion.
17. Pellet the DNA by centrifigation (14000 rpm, 5 min).
18. Rinse the pellet with 1 ml ice cold 70% ethanol.
19. Remove all ethanol carefully with a Pasteur pipette and resuspend the DNA pellet in 100 µl TE.
20. Add 5 µl RNase.
21. Store the DNA at 4 °C.

Random mutagenesis. A vector containing a chromosomal DNA fragment can inactivate a gene after integration in the chromosome by homologous recombination if the fragment is an internal part of that gene. In the pORI vector, pORI19, random chromosomal DNA fragments can be cloned in the mcs in α*lacZ* (Figure 4). The vector allows easy assessment of the cloning efficiency by screening for α-complementation in the $RepA^+$ *E. coli* EC101. The pORI19 library is subsequently integrated in *L. lactis* MG1363 with the help of the temperature sensative plasmid pVE6007, which is used to uncouple the transformation and integration events (Figure 5). The same temperature sensative plasmid, pVE6007, is used to excise and rescue the integration plasmid from an identified mutant (Figure 6).

A. Preparation of media and solutions

1. GM17E[5]C[5] growth medium: dissolve 3.7 g M17 in 100 ml ddH_2O, sterilize (15 min 120 °C) and store at room temperature in the

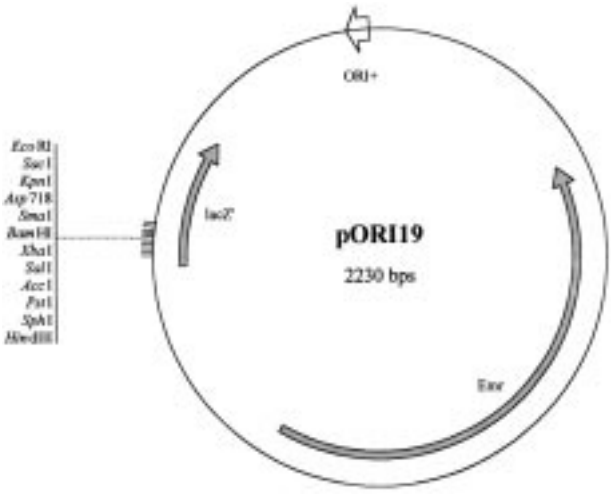

Figure 4. Plasmid map of pORI19 (2.3 kb). The restriction enzyme recognition sites indicated are unique.ORI+ (open arrow): origin of replication of pWV01; lacZ': gene encoding the α-fragment of LacZ; Emr: erythromycin resistance gene.

dark. Add 2.5 ml of a sterile 20% glucose solution, 50 µl of an erythromycin solution (10 mg/ml) and 50 µl of a chloramphenicol solution (10 mg/ml) just prior to use.

2. GM17E^{5}C^5 agar medium: as in 1 but add 1.5 g agar before sterilization.
3. GSM17E^{5}C^5 agar medium: as in 2 but add 10 g sucrose before sterilization.
4. Chloramphenicol (10 mg/ml): dissolve 1 g chloramphenicol in 100 ml ethanol. Store at –20 °C.

B. Random mutagenesis in MG1363 using pORI19 [10]

1. Insert a pool of chromosomal fragments ranging in size from 500 to 1,500 bp in a suitable restriction enzyme recognition site of the pORI19 mcs (Figure 4) and transform to *E. coli* EC101 (RepA$^+$) (Figure 5). Check the efficiency of the ligation by: (i) screening for α-complementation; more than 90% of the colonies should be white or light blue; (ii) confirming the presence and determining the length of the inserts in 100 colonies by PCR, using standard pUC sequencing primers; the average insert length should be within 500 to 1500 bp. Calculate the number of colonies (N) that is required to have 99% probability (P = 0.99) of having a given DNA sequence in the library:

   $N = \ln(1 - P)/\ln(1 - f)$, in which f is the average size of the insert divided by the size of the genome (2.5×10^6 bp for *L. lactis*).

   Note: the chromosomal DNA fragments can be generated by using a restriction enzyme that generates a majority of fragments smaller than 500 bp. By varying the enzyme reaction conditions such as time, temperature or salt concentration, the desired pool of partially digested fragments can be obtained [26]. Alternatively, the chromosomal DNA can be broken by using mechanical shearing techniques. In *L. lactis*, fragments smaller than 500 bp result in very low recombination frequencies [2]. On the other hand the fragments should not be too big, since only a DNA fragment internal to a gene will inactivate that gene upon integration of the vector.
2. Flood the plates with the *E. coli* colonies containing the pORI19 library with 2 ml *E. coli* growth medium each. Scrape the cells from the plates using a glass rod. Pool the cells from all plates in one tube.
3. Isolate the plasmid content without further propagation of the library.
4. Use 0.1 to 1 µg of the pORI19 library (maximum volume: 3 µl in TE) to electrotransform *L. lactis* MG1363 (pVE6007).
5. Incubate the transformation mixture for 90 min at 30 °C in the presence of 50 ng erythromycin to induce expression of the Em^r gene of pORI19 (step 6 in the protocol for the electroporation as described under the procedures for silent gene replacements, paragraph C).
6. Increase the erythromycin concentration in the transformation mixture to 5 µg/ml and incubate for a further 90 min at 30 °C to ensure proper replication of the plasmid bank.
7. Incubate the transformation mixture at 37 °C for at least 3 h to stop replication of pVE6007 and the pORI19 library.
8. Select transformants on GSM17E^5 agar plates and incubate overnight at 37 °C.
9. Transfer the plates to 30 °C and incubate for another 24 h. Colonies should now be visible.
10. Isolate chromosomal DNA from cultures of a number of colonies to check for random insertion by Southern hybridization.
11. Flood the plates with the *L. lactis* library of integrants with 2 ml GM17 medium each and collect all cells in one tube.
12. Centrifuge at 6,000 rpm for 5 min.
13. Discard the supernatant and replace it with the same volume of GM17.
14. Add glycerol to an end concentration of 10%.
15. Store aliquots of 1 ml at –80 °C.

C. Screening of mutants and rescue of the integration plasmid (Figure 6)

1. Thaw an aliquot of the *L. lactis* library of integrants and plate dilutions that enable to discriminate between the wild-type and the mutant phenotype.
2. After identification of a mutant, prepare

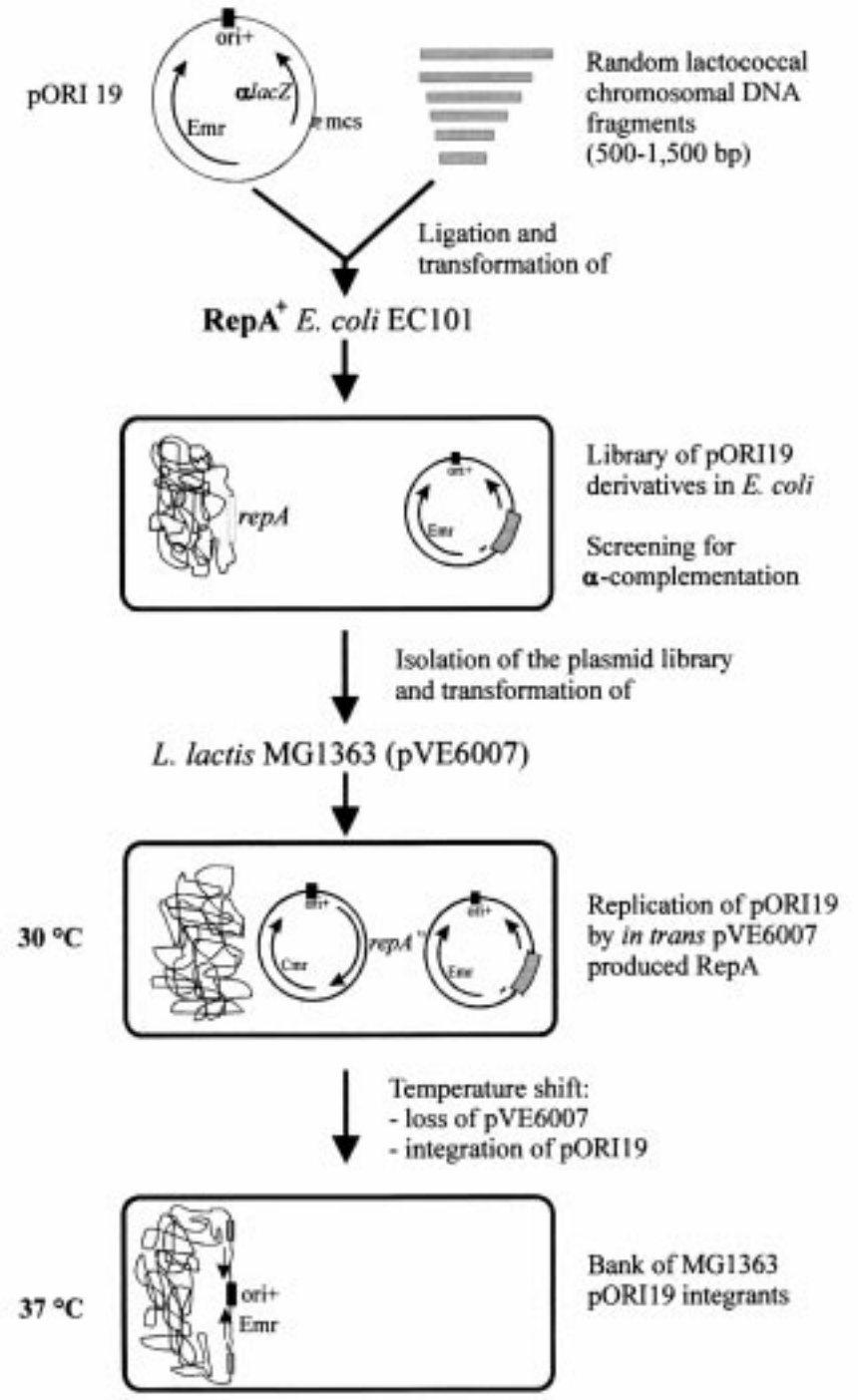

Figure 5. Scheme of using pORI19 for generating random chromosomal insertions in *L. lactis*. In the first step (I) a library of pORI19 containing random lactococcal chromosomal DNA fragments is established in *E. coli* EC101 ($RepA^+$). In the second step (II) *L. lactis* MG1363 (pVE6007) is transformed with the pORI19 library. The integration of the pORI19 derivatives is effectuated by making use of the temperature-sensitive character of pVE6007 ($RepA^{ts}$). See text for details.
ORI+ (black box): origin of replication of pWV01; Em^r: erythromycin resistance gene; α*lacZ*: gene encoding the α-fragment of LacZ; grey boxes: random lactococcal chromosomal fragments; $RepA^+$: strain producing RepA of pWV01; *repA* (open box): gene encoding RepA of pWV01; $repA^{ts}$: gene encoding a temperature-sensitive RepA of pWV01; cm^r: chloramphenicol resistance gene.

mutant competent cells according to the protocol described under silent gene replacements, paragraph B.

3. Introduce pVE6007 in the mutant strain as described under silent gene replacements, paragraph C. Select transformants by growth at 30 °C for at least 48 h on the same type of agar plates that were used to identify the

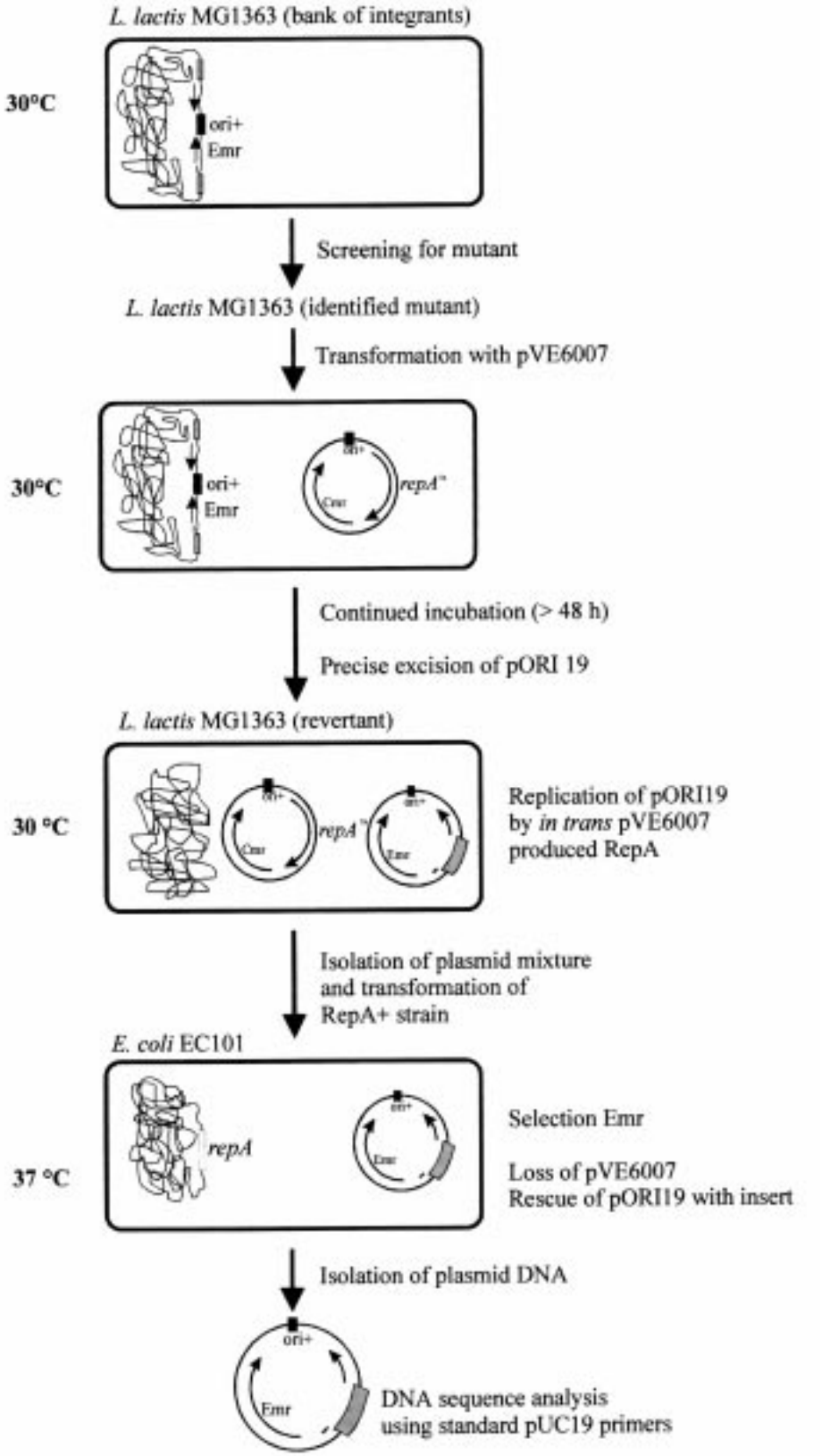

Figure 6. Rescue of an integrated plasmid from an identified mutant using pVE6007 ($RepA^{ts}$). See text for details. ORI+ (black box): origin of replication of pWV01; Em^r: erythromycin resistance gene; α*lacZ*: gene encoding the α-fragment of LacZ; grey boxe: lactococcal chromosomal DNA fragment; $RepA^+$: strain producing RepA of pWV01; *repA* (open box): gene encoding RepA of pWV01; $repA^{ts}$: gene encoding a temperature-sensative RepA of pWV01; cm^r: chloramphenicol resistance gene.

mutant but include chloramphenicol (end concentration 5 μg/ml).
4. Select a transformant that has reverted to the wild-type phenotype and grow the strain overnight in 3 ml GM17E^{5}C^5 at 30 °C.
5. Isolate plasmid DNA according to the 'miniprep' procedure.
6. Use mixture of the two plasmids to transform a RepA$^+$ strain, e.g., *E. coli* EC101.
7. Select only for the presence of the Em^r gene in pORI19 and incubate the plates at 37 °C. In this way pVE6007 is lost.
8. Isolate the pORI19 derivative that contains part of the gene of interest. This plasmid can be used for sequencing with the standard pUC sequencing primers.

D. 'Miniprep' plasmid DNA isolation procedure by means of the alkaline lysis method
1. Inoculate the strain in 3 ml GM17E^{5}C^5 and grow overnight at 30 °C (standing culture).
2. Transfer 2 ml overnight culture to a 2 ml microcentrifuge tube and pellet the cells using a microcentrifuge (14,000 rpm, 3 min).
3. Remove the supernatant. Note: it is important to remove all traces of supernatant.
4. Resuspend the cells in 200 μl lysis solution to which lysozyme has been added (end concentration 5 mg/ml).
5. Incubate 15 min at 50 °C.
6. Add 400 μl SDS/NaOH to the solution and mix well by inversion. The mixture should become clear and should not be stored longer than 5 min.
7. Add 300 μl 3 M NaAc pH5.2 and mix well by inversion.
8. Pellet the precipitate by centrifugation (14,000 rpm, 10 min).
9. Pour the supernant into a clean 2 ml microcentrifuge tube.
10. Add 400 μl phenol to the solution and vortex.
11. Add 400 μl Chl/IAA to the mixture and vortex.
12. Separate the two phases by centrifugation (14,000 rpm, 4 min).
13. Carefully transfer 650 μl of the aqueous (DNA-containing) upper phase to a clean 2 ml microcentrifuge tube. Do not take any of the interphase and/or phenol phase.
14. Add 1.3 ml ice cold 96% ethanol to the DNA solution and vortex.
15. Pellet the DNA by centrifugation (14,000 rpm, 10 min).
16. Remove the ethanol using a Pasteur pipette.
17. Rinse the pellet with 1 ml ice cold 70% ethanol.
18. Centrifuge 1 min at 14,000 rpm.
19. Remove the ethanol using a Pasteur pipette.
20. Dry the pellet using a vacuum dryer.
21. Dissolve the DNA in 20 μl TE.
22. Add 1 μl RNase and incubate 10 min at 37 °C.
23. Store at –20 °C.

Random transcriptional fusions. In most cases promoter screening systems are plasmid-based. Multiple copies of a regulated promoter on a plasmid may interfere with the regulation mechanism by titration of regulatory proteins. In addition, plasmid copy numbers may vary. Therefore, we developed the integrative pORI13 transcriptional fusion vector which allows to assess the expression of (regulated) genes in a single copy fusion with a reporter gene in the chromosome. The strategies to obtain a library of pORI13 integrants and the rescue of the integration plasmid from an integrant of interest are the same as described for random mutagenesis (Paragraphs B and C; Figures 5 and 6).

A. Preparation of media and solutions
As described under silent gene replacements and random mutagenesis.

B. Random transcriptional fusions in MG1363 using pORI13 [29]
1. Insert a pool of chromosomal DNA fragments in a suitable restriction enzyme recognition site of the pORI13 mcs (Figure 7) and transform *E. coli* EC1000 (RepA$^+$). This strain lacks *lacZ*. Generate the fragments in a similar way as described for the random mutagenesis procedure paragraph B. It is not necessary to use small chromosomal fragments to obtain transcriptional fusions after integration of the plasmid in the chromosome, as large fragments also may result in transcriptional fusions so long as no transcriptional terminator is present between the promoter and the reporter gene. The chromosomal fragment does not have to carry a promoter sequence. If the fragment carries only part of an open reading frame, the reporter gene in pORI13, after the integration event, will be placed under the control of the promoter present in the upstream chromosomal DNA.
2. Isolate the pORI13 library and follow the procedures of the random mutagenesis (paragraph B) to establish and stock a library of pORI13 integrants.

C. Screening for environmentally regulated genes and rescue of the integration plasmid
1. Thaw an aliquot of the *L. lactis* library of integrants and plate appropriate dilutions onto X-gal containing plates. Apply an environmental condition that will activate a certain set of genes.
2. Select blue colonies and transfer them to GM17E^{5}Xgal plates and incubate at 30 °C.
3. Identify colonies that are white or light blue under 'normal' conditions (GM17E^{5}Xgal at

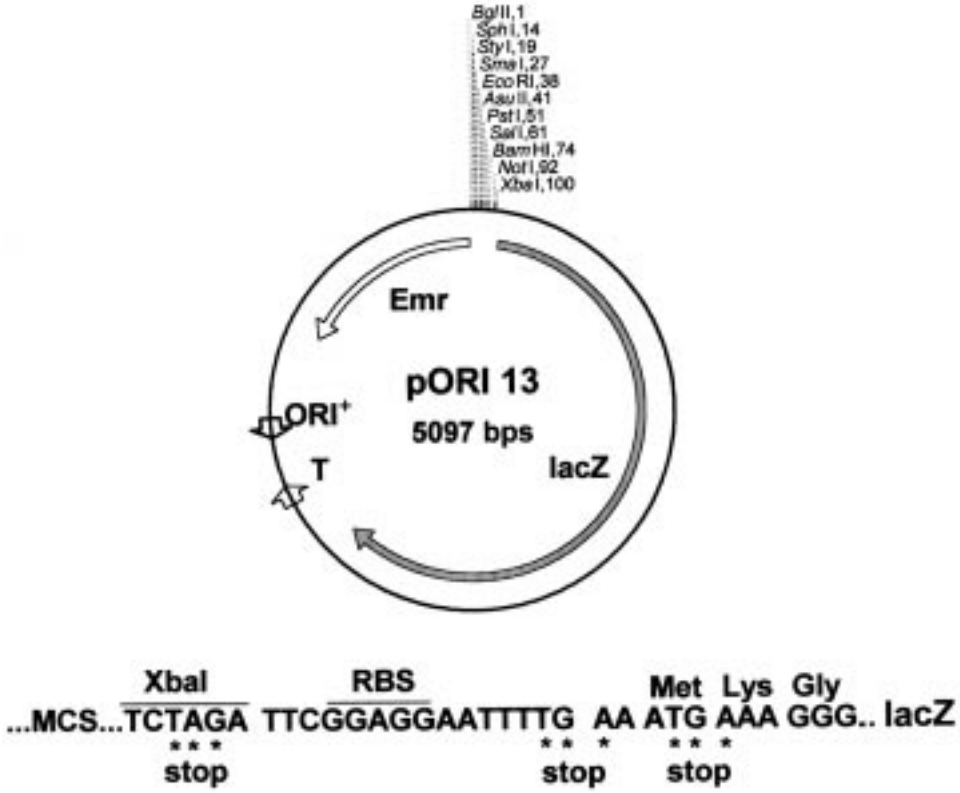

Figure 7. Plasmid map of pORI13 (5.1 kb). ORI+ (open arrow): plus origin of replication of pWV01; Em^r: erythromycin resistance gene; T (open arrow): terminator of *prtP*; *lacZ*, promoterless *E. coli* β-galactosidase gene fused to the ribosome binding site (RBS) and translational start codon of lactococcal *orf-32* (shown in detail). Stop codons are indicated by asterisks. Unique restriction enzyme recognition sites present in the multiple cloning site (MCS) are shown.

30 °C) and that are blue under 'stress' conditions.

4. Excise and rescue the integration plasmid from the integrant of interest as described before (random mutagenesis, paragraph C).

4. Results and discussion

Silent gene replacements. The vector pORI280 (Figure 2) has been constructed to replace genes in a two-step strategy and allows the isolation of mutants which do not carry a selectable marker (Figure 3). The mcs of pORI280 contains several unique restriction enzyme recognition sites that allow insertion of chromosomal DNA fragments that flank the gene to be deleted or mutated. For *L. lactis* a minimum size of 500-bp for each flanking region is recommended. It has been observed that recombination frequencies in *L. lactis* increase when the sizes of the chromosomal fragments increase, although no further stimulation of recombination was observed for fragments larger than 2.5-kb [2]. To detect resolution of plasmids integrated in genes for which no simply detectable phenotypic difference exists between the wild-type and mutant copy, the *E. coli lacZ* reporter gene is present in pORI280. The *lacZ* gene is under control of the lactococcal promoter P_{32} which is recognized in Gram-positive and Gram-negative bacteria. Strains containing an integrated copy of pORI280 are erythromycin resistent (Em^r) and stain blue on agar plates containing X-gal. After excision of the plasmid from the chromosome by homologous recombination through one of the flanking regions, the *lacZ* reporter and Em resistance genes (and Ori^+-fragment) are lost. Consequently, colonies which arise from cells in which this process has occurred stain white on non-selective agar plates containing X-gal, a readily scorable phenotype among the majority of blue colonies which retained the integrated plasmid. Resolution of the integrated plasmid either restores the wild-type gene or results in a gene replacement. Standard DNA analysis techniques or, if available, an activity assay can be used to discriminate between the two possibilities.

To illustrate the feasibility of the strategy, integration studies using the *pepX* X-prolyl-dipeptidyl amino peptidase) gene region of *L. lactis* MG1363 will be described. We selected this region because in previous gene-replacement studies we observed that recombination frequencies in *pepX* of this strain are relatively low with values as low as 10^{-6} per generation. Two 1.5-kb fragments of *pepX* 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-*pepX*, *pepX* lacks an

internal 716-bp fragment. pORI280-*pepX* was used to transform *L. lactis* MG1363 by the two-step strategy outlined in Figure 2. Colonies were screened for LacZ activity but not for PepX activity during this procedure to mimic the case in which a bioassay is lacking. We first determined through which fragment pORI280-*pepX* had integrated. Southern blots were used to analyse 28 transformants: nineteen had integrated through fragment A (MG1363::*pepX-A*) and nine through fragment B (MG1363::*pepX-B*). One transformant of each type was taken and grown for 35 generations under nonselective conditions to allow resolution of the cointegrate structure. Approximately 20,000 colony forming units (4,000 cfu per ∅ 15 cm plate) of each culture were plated onto agar medium containing X-gal. Five $LacZ^-$ colonies were detected among the MG1363::*pepX-A* colonies and nine among those derived from MG1363::*pepX-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::*pepX-A* and MG1363::*pepX-B* cultures, respectively. The mutant nature of the colonies was confirmed by the PepX plate assay. Taken together, the frequencies by which gene replacements were generated in the MG1363::*pepX-A* and MG1363::*pepX-B* cultures ranged between 1.4×10^{-6} and 4.2×10^{-6} per generation. Recombination through the promoter-containing fragment A was consistently more efficient than through the promoterless fragment B. We also observed this phenomenon in other replacement studies and it is in agreement with results described earlier by Biswas et al. [2].

The pORI280-system has been used in our group to replace over ten different chromosomal genes. The recombination frequencies observed for these genes range from approximately 10^{-4} to 10^{-5} per generation. We noted that recombination in the second step of the procedure could be stimulated about five to ten times by increasing the growth temperature to 37 °C, a temperature that induces a heat-shock response in *L. lactis*. The system has also been successfully applied to combine several different gene replacements in a single strain. For instance, a strain was constructed from which seven different peptidase genes were removed [20, M. A. Hellendoorn, unpublished data]. During this work it was observed that the promoter P_{repC} of pORI280 is active and can drive the transcription of genes located downstream of the plasmid insertion site (Figure 3). This is an important feature if the gene to be removed from the chromosome is located upstream of (an) essential gene(s) in an operon structure. Thus, polar effects due to the insertion of the pORI vector can be avoided by employing the transcriptional activity of P_{repC} [14].

The pORI280-system has also been successfully used in *B. subtilis* [14]. It is very likely that the vectors can be used in many other bacterial hosts. An exception may be some (lactococcal) strains harbouring pWV01-like plasmids, as these would provide RepA *in trans*. In such a case, a plasmid curing step prior to the use of pORI280 will be necessary. Adjustment of the vector 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. A pORI-vector with a tetracycline resistance marker, pORI240, is available. Other adjustments can easily be achieved by virtue of the modular design of the vectors. The pORI plasmids have the advantage over other nonreplicative 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 which are sufficiently high to obtain at least a few integrants. Therefore, it may be necessary to optimise transformation protocols for poorly transformable strains.

The pORI280 system uses *lacZ* expression to visualize recombination events in the second step of the procedure by a simple blue/white screening of colonies. As this system is based on negative selection, it may result in extensive screening of colonies if recombination frequencies in the target gene are very low (< 10^{-6} per generation). The reporter gene may be replaced by a gene which allows positive selection, if available. Nevertheless, the pORI280-system was found to be highly efficient in all gene replacements so far. 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 open the possibility to introduce more desired mutations (or genes) in the strain, which is of special interest for metabolic pathway engineering purposes.

Random mutagenesis. A pORI-system suitable for generating random chromosomal insertions makes use of pORI19 (Figure 4). This vector contains an Em^r gene as selectable marker and the pUC19 *lacZ*α gene and mcs in which random chromosomal DNA fragments can be inserted. To inactivate as many genes as possible upon integration of the plasmid bank, a few requirements with respect to the plasmid bank have to be met: (i) the inserts in the plasmid should be relatively small. A gene will be inactivated only when the fragment used for the homologous recombination is internal to the gene. Since the average size of genes is about 1 to 1.5 kb, the cloned chromosomal DNA fragments should preferentially be smaller. However, recombination frequencies of fragments smaller than 500 bp are very low in *L. lactis*. Therefore, DNA fragmentation conditions should generate fragments in the range of 500 to

1,500 bp. This can either be done by using a proper DNA restriction enzyme or by DNA shearing techniques; (ii) ligation of the random chromosomal fragments in the integration vector should be efficient. For this purpose *lacZ*α is present in the vector which allows rapid assessment of the cloning efficiency by testing part of the ligation mixture for α complementation in the $RepA^+$ derivative of *E. coli* JM101, strain EC101 (Figure 5).

For fragmentation of chromosomal DNA of *L. lactis* MG1363 the restriction enzyme *Alu*I was selected. Several partial *Alu*I digests were made in which most fragments ranged in size from 100–1,500 bp. These digest were pooled and the mixture was ligated into the dephosphorylated *Sma*I site of pORI19 and used to transform *E. coli* EC101. More than 90% of the transformants were white or pale blue on agar plates containing Xgal. All of the white colonies analysed contained inserts, as did several of the blue and pale blue colonies, indicating that in-frame insertions had occured in those cases. By PCR, the estimated average insert size in pORI19 of 100 randomly picked colonies was 650 bp. The number of colonies required for 99% certainty that all 0.65-kb fragments of the *L. lactis* MG1363 chromosome had been cloned is 18,000. The plasmid bank was isolated from approximately 30,000 *E. coli* colonies without further propagation of the cells.

Alternatively, the ligation mixture can be introduced in a $RepA^+$ helper strain of one of the other bacterial species to isolate the pORI19 plasmid bank. *E. coli* EC101 can then be merely used to assess the cloning efficiency. One reason for following the latter approach is that in a homologous system (in this case a $RepA^+$ *L. lactis*) a pORI19 library would probably be more complete since it is likely that many lactococcal DNA fragments can not be cloned in *E. coli*. However, we reasoned that a high percentage of those fragments which are unclonable in *E. coli* probably contain (strong) promoters or complete (lethal) genes. Such fragments will not result in mutants since the cloned fragments need to be internal to a transcriptional unit to result in a mutant phenotype. Therefore, constructing the library in the $RepA^+$ *E. coli* strain may increase the percentage of plasmids containing an internal gene fragment, which would enhance the efficiency of the library to generate mutations.

In the next step, the pORI19 plasmid bank was used to transform the $RepA^-$ *L. lactis* strain MG1363. Under optimal conditions this resulted in approximately 10^3 Em^r colonies per μg of pORI 19 plasmid bank DNA. Southern hybridization analysis of chromosomal DNAs of several of the transformants revealed that all carried an integrated copy of pORI19 at different locations. A library of pORI19 integrants in which a maximum number of different genes are inactivated should contain at least 18,000 colonies, if not more. To increase the number of integrants, the transformation event can be separated from the integration event by using a Ts version of a pWV01 derivative which enables the replication of both the Ts and the pORI plasmid at the permissive temperature (see above). Therefore, MG1363 containing the pWV01 Ts-variant pVE6007 (Cm^r) was transformed with the pORI19 plasmid bank (Figure 5). A subsequent temperature shift from 30 °C to 37 °C causes loss of pVE6007 and integration of the pORI19 derivatives at the sites on the chromosome from which the inserts originated. An 1-μg sample of the pORI19 chromosomal DNA bank was used to transform *L. lactis* MG1363 (pVE6007). The transformation mixture was incubated at 30 °C for 90 min in the presence of 50 ng of Em per ml to induce expression of the Em^r gene. Subsequently, the Em concentration was increased to 5 μg/ml and incubation was continued at 30 °C for a further 90 min to ensure proper replication of the plasmid bank in *L. lactis* prior to the temperature shift. Plating the bank at this point and incubating overnight at the non-permissive temperature for pVE6007 (37 °C) did not cure the plasmid, as 40% of the colonies at this stage were Em^rCm^r and thus harbored both plasmids. To ensure total curing of pVE6007, it was necessary to incubate the transformation mixture at 37 °C for at least 3 h following the 3 h period at 30 °C before plating on GM17 Em agar plates and incubation overnight at 37 °C. Overnight incubation at 30 °C following this 6-h treatment reduced the percentage of colonies harboring both replicating plasmids to 10%, while at 37 °C all Em^r colonies, recovered at a frequency of 10^4/μg of DNA, were cm^s. The chromosomal DNAs of several integrants were analysed in Southern hybridizations and these indicated that pORI19 had integrated at different sites [10].

To assess the feasibility of the system to generate stable random chromosomal mutations in *L. lactis*, the bank of integrants was screened for a mutation in the cell wall hydrolysing system. The target gene, *acmA*, was selected because: (i) it is nonessential; (ii) a simple bioassay is available, and (iii) the gene is monocistronic and of average size (1.3 kb). The size of the target fragment is even smaller if it is taken into account that the three repeats located in the C terminus of the hydrolase can be removed without loss of enzyme activity. Therefore, integration within the first 700 bp of the gene is required in order to inactivate it. About 5000 *L. lactis* transformants were spread onto glucose-M17 plates in which autoclaved *Micrococcus lysodeikticus* cells had been included, allowing approximately 40 colonies per plate. One transformant without a halo was detected and analysis of its chromosomal DNA learned that, indeed, pORI19 had integrated in an internal 5′-fragment of *acmA*.

The pORI19 system was further tested by selection of mutants in the maltose metabolic pathway, as isolation of stable Mal^- mutants had previously been shown to be unsuccessful using other insertional

methods. The bank of lactococcal integrants was plated on maltose indicator plates with a density of approximately 1000 colonies per plate. Following 24 hours of incubation at 30 °C, 1 in 10,000 colonies was found to be unable to ferment maltose (approx. 30,000 colonies were screened). One of the *L. lactis* Mal^- colonies was streaked for single colonies on maltose selective agar and after 3 days of incubation at 30 °C, all of the colonies were still white, indicating that the mutation was stable. A culture of this strain maintained its maltose-negative phenotype even after incubation overnight at 30 °C in GM17 without Em and subsequent plating on maltose selective agar.

The maltose-negative strain was used to develop a strategy for the easy isolation of the integrated plasmid from a defined mutant. Such a strategy would allow to rapidly identify the disrupted gene (Figure 6). *L. lactis* Mal^- was endowed with pVE6007 ($RepA^{ts}$) and plated on maltose indicator agar containing Cm and Em. After 24 h of incubation at 30 °C all transformants were still unable to ferment maltose, whereas after 48 h approximately 8% were faintly yellow, indicating that cells in these colonies had reverted to wild type. After colony purification it was found that 20% had regained maltose-fermenting ability, most probably by precise excision of the integrated plasmid. A Mal^+ *L. lactis* single colony isolate contained two plasmids, pVE6007 and pORI19 containing an insert (pMAL). To rescue pMAL, the plasmid mixture was used to transform *E. coli* EC101 ($RepA^+$) at 37 °C with selection for Em^r only, resulting in the separation of pVE6007 and pMAL. Upon introduction of pMAL into *L. lactis* MG1363 ($RepA^-$), all integrants were maltose negative, as expected. Southern hybridisation analysis of five transformants revealed that pMAL had integrated at the same site on the lactococcal chromosome as in *L. lactis* Mal^-.

The nucleotide sequence of the *Alu*I insert in pMAL was determined by making use of the standard pUC19 sequencing primers. A continuous open reading frame was present on this 564-bp fragment, and its deduced amino acid sequence of 189 amino acids showed high homology to ATP-binding proteins of several sugar uptake systems, among which the inner membrane MalK proteins of the maltose uptake systems of *Enterobacter aerogenes* and *E. coli*. These results suggested that, indeed, a gene involved in the maltose metabolic pathway of *L. lactis* had been targeted and that part of it had been cloned [10]. The 5′- and 3′-end of the gene may now be cloned by restriction of the chromosomal DNA of the Mal^- integrant with suitable enzymes and recovering the integration plasmid with additional flanking DNA in one of the $RepA^+$ helper strains.

Although the method described here is efficient in generating chromosomal insertions, the isolation of genes is, like most insertional methods, restricted to non-essential ones and to genes that allow detection of phenotypic negatives. Another disadvantage of the method is that rather high transformation frequencies are required if the Ts-helper plasmid can not be used, which may be the case in a number of bacterial species. Critically important is also the construction of a proper pORI19 library in one of the $RepA^+$ helper strains. However, cloning efficiencies can be easily assessed by the availabilty of the *E. coli* helper strain EC101. Other important advantages of the method are that: (i) stability of the mutants is high because of the absence of residual activity of transposases or (Ts) replication proteins; (ii) screening of mutants can be performed at optimal growth temperatures; (iii) the integration plasmid can be readily recovered by a simple and rapid procedure, and (iv) the availability of $RepA^+$ *L. lactis*, *B. subtilis*, and *E. coli* helper strains minimizes difficulties (lethality and deletions) in cloning of the targeted gene in incompatible host backgrounds.

Random transcriptional fusions. An Ori^+-integration vector, pORI13, was developed to screen for (environmentally regulated) gene expression signals in *L. lactis* and to assay for transcriptional activity of genes in single copy. The plasmid carries a promoterless *E. coli lacZ* gene preceded by a start codon, a lactococcal ribosome binding site, and a mcs. It contains translational stop codons in all three reading frames upstream of or overlapping the start codon of *lacZ* to prevent translational fusions (Figure 7). Plasmid pORI13 did not produce detectable β-galactosidase activity when replicating in *L. lactis* $RepA^+$ helper strains.

To produce chromosomal transcriptional fusions using pORI13 there are no restrictions with respect to the size of the fragment and, also, promoter elements do not have to be present on the cloned chromosomal fragment. Any fragment of a transcriptional unit cloned upstream of *lacZ* will produce a transcriptional fusion after integration of pORI13, provided that the transcriptional terminator of that unit is lacking. Several partial *Sau*3A digests of *L. lactis* MG1363 chromosomal DNA, with the majority of the fragments ranging in size from 1 to 10 kb, were pooled and the mixture was ligated into the alkaline phosphatase-treated *Bam*HI restriction enzyme site of pORI13. The ligation mixture was used to transform *E. coli* EC1000 ($RepA^+$), a derivative of strain MC1000 (Δ*lacZ*). More than 10,000 transformants were collected from agar plates and their plasmid DNA content was isolated. The resulting pORI13 plasmid DNA bank was used for integration in the chromosome of *L. lactis* MG1363 by employing the same strategy as described for pORI19, with pVE6007 as a means to uncouple transformation and integration events. The system was tested by plating the transformants, immediately after the temperature shifts, onto selective agar plates containing 0.3 M

NaCl as the stress inducing agent. Southern hybridisation analysis of the chromosomal DNAs of more than 10 integrants showed that pORI13 had inserted in all cases at different positions in the chromosome of these clones. One hundred and ninety five colonies showed various levels of blue staining in the presence of NaCl and Xgal after prolonged incubation at 30 °C, which is also indicative of *lacZ* insertion in different transcribed regions.

These colonies were transferred to GM17Xgal agar plates with or without 0.5 M NaCl. Various phenotypes could be distinguished: colonies staining light blue in the absence of NaCl and staining dark blue in the presence of NaCl; colonies which were blue in the absence of NaCl but light blue in the presence of NaCl. One selected clone, designated *L. lactis* NS3, produced blue colonies on plates with NaCl but was white on plates without added NaCl. Interestingly, β-galactosidase activity increased proportionally from 0 to 50 Miller units with NaCl concentrations in the medium increasing from 0 to 500 mM NaCl. The pORI13 derivative integrated in NS3 (pNS3) was rescued as described above using pVE6007 to excise the plasmid from the chromosome. Plasmid pNS3 still showed the NaCl-dependent phenotype when replicating in *L. lactis* ($RepA^+$), indicating that the regulatory elements were present in the 10-kb insert upstream of *lacZ* [29]. Detailed sequence and biochemical analyses learned that *lacZ* had been transcriptionally fused to an operon that is regulated by chloride ions, glutamate and low pH. Furthermore, the salt inducible promoter is preceded by a constitutively expressed gene encoding a positive regulator which acts on the salt inducible promoter in a still unknown way [27].

The results described above demonstrate that pORI13 can be used to study environmentally regulated gene expression signals in a single copy situation. The relatively large size of the chromosomal DNA fragments used for integration increases the probability that, upon integration of pORI13, in addition to the disrupted copy, a wild-type gene continues to be present in the integrant. pORI13 can also adventageously be used to trace transcription regulatory elements: after integration via the 3′-end of an operon, *lacZ* will be under control of a promoter upstream of the fragment used for recombination. The pORI13 system is suitable both for single copy screening of regulated promoters and the construction of targeted transcriptional fusions without gene disruption [28].

In conclusion, we have shown that the combined use of two conditionally replicating vectors derived from the same broad-host-range plasmid pWV01 is an important and extremely valuable research strategy in the analysis of the lactococcal chromosome. Also, we strongly believe that the vectors described here may represent useful tools in other species of bacteria.

Acknowledgments

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Notes on suppliers

1. Acros Organics, B-2440 Geel, Belgium
2. Becton Dickinson and Co., Cockeysville, MD 21030, USA
3. BDH Laboratories, Poole BH15 1TD, England
4. BIORAD Laboratories, Hercules California, CA 94547, USA
5. Boehringer Mannheim GmbH, Mannheim, Germany
6. Difco Laboratories, Detroit, MI 48232-7058, USA
7. Eppendorf- Netheler-Hinz-GmbH, D-22331 Hamburg, Germany
8. EuroGenTec, B-4102 Seraing, Belgium
9. Greiner GmbH, D-7443 Frickenhausen, Germany
10. Lab-Scan Ltd., Dublin, Ireland
11. Leenhouts K., Biological Centre, Department of Genetics, Kerklaan 30, NL-9751 NN Haren, The Netherlands
12. Maguin E., Génétique Microbienne, INRA, F-78352 Jouy en Josas, France
13. Merck, D-64271 Darmstadt, Germany
14. Omnilabo international, NL-4800 DX Breda, The Netherlands
15. Phoenix Biomedical, Ontario L5S 1R7, Canada
16. Sartorius Filtratie BV, NL-3439 MN Nieuwegein, The Netherlands
17. Savant Instruments Inc., Farmingdale, NY, USA
18. Sigma Chemical Co., St. Louis, MO 63178-9916, USA

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