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pSEUDO, a Genetic Integration Standard for *Lactococcus lactis*†

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Plasmid pSEUDO and derivatives were used to show that *llmg_pseudo*10 in *Lactococcus lactis* MG1363 and its homologous locus in *L. lactis* IL1403 are suitable for chromosomal integrations. *L. lactis* MG1363 and IL1403 nisin-induced controlled expression (NICE) system derivatives (JP9000 and IL9000) and two general stress reporter strains (NZ9000::P*Phuc4*-GFP and NZ9000::P*groES*-GFP) enabling in vivo noninvasive monitoring of cellular fitness were constructed.

*Lactococcus lactis* is a widely used Gram-positive model organism that belongs to the clade of lactic acid bacteria (LAB). Advances in the genomics research of *L. lactis*, such as the sequencing of the genomes of the most commonly studied subspecies (1, 14, 23) and the various genome-wide transcriptome studies (see, e.g., reference 4), provide a broad view of the mechanisms of genetic regulation in this economically important organism. This knowledge is invaluable for strain selection and for the improvement of lactococcal strains by rational design. However, a standard locus in the chromosome of *L. lactis* for integration of DNA fragments, whether for genetic complementation (single-copy or merodiploid-like situations) or cloning of a reporter gene or promoter, is lacking. Whereas, for example, in Bacillus subtilis the amyl locus is often used for these purposes (19), in *L. lactis* various loci in the chromosome have been chosen as targets for integration. Unfortunately, to the best of our knowledge none of the proposed strategies exclude the possibility of phenotypic consequences. The *leuA* locus was shown to suffer from active read-through from the native *leuA* promoter (10), whereas the choice of the sex factor locus might interfere with the biology of *L. lactis* and, in addition, may have consequences with respect to possible conjugal transfer of the inserted DNA. Also, bacteriophage sequences have been used to drive site-specific integration of plasmids in the chromosome of *L. lactis* (2, 22). However, these methods do not allow making strains without resistance markers, while some require the use of a second plasmid to provide the bacteriophage integrase in trans. Furthermore, the localization in the chromosome of *L. lactis* MG1363 of sequences with high similarity to that at a given *attB*, e.g., in the *comGC* gene for the TP901-1 *attB* and in *rex* for the TUC2009 *attB* (data not shown), implies that the integration process might lead to the simultaneous disruption of potentially relevant processes.

Here it was examined whether the *llmg_pseudo*10 locus of *L. lactis* MG1363 or its corresponding region in *L. lactis* IL1403 is a suitable neutral region for chromosomal integrations (Fig. 1). In *L. lactis* IL1403, this locus contains *yfjF*, a gene of 1,506 bp whose product exhibits homology to transport proteins from the major facilitator superfamily. In *L. lactis* MG1363, translation is halted prematurely due to the presence of a stop codon at position 303, hence its annotation as a pseudogene (23). By cloning and resequencing, the nucleotide sequence of this region in *L. lactis* MG1363, originally described by Wegmann et al. (23), was confirmed. The loss of function of the locus in *L. lactis* MG1363 suggests that *yfjF* is nonessential. The *llmg_pseudo*10 locus has been shown to be silent throughout the growth of *L. lactis* MG1363 in batch cultures in M17 medium (R. W. W. Brouwer, J. P. C. Pinto, A. Zeinyiev, J. Kok, and O. P. Kuipers, submitted for publication) and milk (A. de Jong, personal communication). In addition, *llmg_pseudo*10 and *yfjF* display low nucleotide sequence similarity with other regions in the *L. lactis* genome, minimizing the possibility of illegitimate recombination.

Construction and applicability of pSEUDO and pSEUDO-GFP. To be able to perform unmarked integrations in the chromosome of *L. lactis*, the chromosomal integration vector pCS1966 was employed, allowing positive selection of cells in which the plasmid had been excised from the genome (20). Two DNA fragments were amplified from *L. lactis* MG1363 chromosomal DNA by PCR, one of 529 bp, obtained with the primer pair P1_pseudo10/P2_pseudo10, and another of 804 bp, obtained using P3_pseudo10/P4_pseudo10, and sequentially inserted into pCS1966 using the restriction enzymes indicated in Table 1 and Escherichia coli DH5α as the cloning host. Selection was performed on tryptone-yeast extract (TY)-agar plates with 150 μg/ml erythromycin. The custom-made multiple cloning site GAAATTCCCGCGATGCCGCGGTGCAC AAGCTTAGATCTCGAGGATCC was introduced between the BamHI and EcoRI sites, thus producing the plasmid pSEUDO (Fig. 1). This vector can be used to insert DNA fragments into the *llmg_pseudo*10 locus of *L. lactis* MG1363 using positive selection for resistance to the toxic pyrimidine analog 5-fluoroorotate by methods described before (20), with minor modifications (18). Although it is not possible to screen for integrants via loss of function through gene disruption, pSEUDO allows for a quick and efficient positive survival
strategy to monitor both integration (erythromycin resistance) and excision of the vector backbone from the chromosome (5-fluoroorotate resistance), thus enabling the production of unmarked strains in an easy and fast manner.

As a proof of principle, the applicability of pSEUDO is illustrated through the integration, internal and in opposite orientation to lmg_pseudo_10, of the genes of the two-component nisin sensor, NisRK (11), in the chromosome of L. lactis MG1363. A fragment containing the nisRK genes with their own promoter was amplified by PCR from chromosomal DNA of L. lactis NZ9000 (19) using the primers nisRK_Forw and nisRK_Rev. The PCR product and pSEUDO were digested with BamHI and ligated after dephosphorylation of the digested vector, producing pSEUDO::nisRK. The presence and orientation of the insert were checked with PCR and restriction endonuclease digestions. The integration of nisRK into L. lactis MG1363 using pSEUDO::nisRK generated the L. lactis strain JP9000. Expression of nisRK and the applicability of the nisin-induced controlled expression (NICE) system (6, 11) using L. lactis JP9000 are the same as for L. lactis NZ9000. As an example, the NICE system was used for the overproduction of a green fluorescent protein (GFP)- and hexahistidine-tagged membrane protein of L. lactis, BcaP-GFP-H6, using pNZ8048 derivative in which the bcaP-gfp-H6 gene is driven by the nisin-inducible PnisA promoter (12). L. lactis strains NZ9000 and JP9000 carrying this plasmid produced equivalent amounts of the tagged protein since similar fluorescent signals from the overproduced BcaP-GFP-H6 were obtained using either strain (Fig. 2). Contrary to previous observations (10), the native nisRK promoter was sufficient to yield significant amounts of NisRK and read-through from the neighboring genes was not required for a functional NICE system. In addition to pSEUDO::nisRK, a similar vector was constructed for nisRK integration in the yfjF locus of L. lactis IL1403. The lmg_pseudo_10 homologous regions of pSEUDO were replaced by homologous regions of the yfjF locus, amplified from chromosomal DNA of IL1403 using primers P1_yfjF, P2_yfjF, P3_yfjF, and P4_yfjF (Table 1). The nisRK genes were inserted in the BamHI site in opposite direction to the yfjF gene, resulting in plasmid pSEUDO::nisRK. The integration of nisRK into L. lactis IL1403 resulted in strain IL9000. To test functionality, IL9000 was transformed with a pNZ8048-derived plasmid in which gfp was inserted downstream the nisin-inducible PnisA promoter (H. Trip, unpublished results). GFP fluo-

![FIG. 1. Genomic context of lmg_pseudo_10 of L. lactis MG1363 and its relation to the homologous regions (hr’s) present in pSEUDO and pSEUDO-GFP. The multiple cloning site (MCS) contains, in clockwise order, EcoRI, XmaI/SmaI, SphI, ScaI, Sall, HindIII, BglII, XhoI, and BamHI restriction enzyme sites. The vertical line on lmg_pseudo_10 depicts the stop codon that prematurely halts translation of the gene in L. lactis MG1363. eryR, erythromycin resistance gene. orope pseudo encodes the orotate transporter (3).](image-url)

### TABLE 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence (5’-3’)*</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1_pseudo10</td>
<td>GCCCTAGAACATAATTGCCCATGCTTGATTCC</td>
<td>BglII</td>
</tr>
<tr>
<td>P2_pseudo10</td>
<td>CGCGGATCCCTTGTTGGGTTAAGGAGTTGAGAGAG</td>
<td>BamHI</td>
</tr>
<tr>
<td>P3_pseudo10</td>
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<tr>
<td>P4_pseudo10</td>
<td>CGCGTACCAATATTGAGAGACAGAAGAAAAAC</td>
<td>KpnI</td>
</tr>
<tr>
<td>P1_yfjF</td>
<td>GCCGAAAATCTAGACTCAAACATAAGAGACCTGG</td>
<td>XbaI</td>
</tr>
<tr>
<td>P2_yfjF</td>
<td>GCCGAAAAGATGCTTTAGCTTATGAGGTTGAAAGG</td>
<td>BamHI</td>
</tr>
<tr>
<td>P3_yfjF</td>
<td>GCCGAAAAGATGCTTTAGCTTATGAGGTTGAAAGG</td>
<td>BamHI</td>
</tr>
<tr>
<td>P4_yfjF</td>
<td>GCCGAAAATCTAGACTCAAACATAAGAGACCTGG</td>
<td>XbaI</td>
</tr>
<tr>
<td>nisRK_Forw</td>
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</tr>
<tr>
<td>nisRK_Rev</td>
<td>AGATTGGAATCCCATACTGATATCCTTGTAGACCTGC</td>
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</tr>
<tr>
<td>GFP-ŠF_Forw</td>
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<td>XhoI</td>
</tr>
<tr>
<td>GFP-ŠF_Rev</td>
<td>ATCATGGGATCTATATAACCCAGAAGAGGCCACC</td>
<td>BamHI</td>
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<tr>
<td>PhrA_forw</td>
<td>ATCTGAGATCTCATACCAGACGTTATTACCC</td>
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<tr>
<td>PgroES_forw</td>
<td>GGAAGGATCTTGGAGAAGCTGAGATGAGAGCCTCTTCTTG</td>
<td>XhoI</td>
</tr>
<tr>
<td>P groES_rev</td>
<td>ATACTGCAGAATTTTATATTGACGACTCTTAAATAG</td>
<td>XhoI</td>
</tr>
</tbody>
</table>

* Restriction enzyme sites are underlined.
FIG. 2. Heterologous protein production and activity of aminopeptidase PepN in *L. lactis*. (A) Expression of the membrane protein BcaP-GFP-H6 was induced in *L. lactis* strains NZ9000 and JP9000, both carrying plasmid pNZbcaP-GFP-H6 (12). The strains were grown in GM17 until an optical density (OD) at 600 nm of 0.5 was reached, after which they were induced for 1 h with 5 ng/ml of nisin. Mean fluorescence, as measured by flow cytometry, of the plasmid-carrying strains normalized to that of plasmid-free *L. lactis* NZ9000 is plotted. The uninduced bar depicts the fluorescence of the noninduced JP9000 (pNZbcaP-GFP-H6) culture. (B) PepN activity in *L. lactis* strains MG1363 (9), NZ9000 (11), MG1363ΔpepN (16), and JP9000 (this work) was determined in cell extracts of cultures grown in GM17 until an OD at 600 nm of 0.5, by monitoring the hydrolysis of the PepN substrate lysyl-p-nitroanilide, as described previously (8). Data (A and B) are the averages of 4 biological replicates, and the error bars are the associated standard deviations.

FIG. 3. Induction of GFP production in *L. lactis* NZ9000::PgroES-GFP and in *L. lactis* NZ9000::PhrcA-GFP grown in GM17 medium at 30°C. Both cultures were shifted to 60°C when they had reached an optical density at 600 nm of 0.5. White, 0 min after the temperature shift; light gray, 15 min after the temperature shift; dark gray, 60 min after the temperature shift. The fluorescence was measured over time using an Epics XL-MCL flow cytometer (Coulter, Fullerton, CA). Values were normalized to the fluorescence of *L. lactis* NZ9000 undergoing the same heat treatment. Twenty thousand cells were measured per experiment, and 4 biological replicates were obtained per strain and per time point. The error bars are the associated standard deviations.

As an example of the applicability of pSEUDO-GFP, promoter-gfp fusions were constructed using the upstream regions of two general stress response genes, *hrcA* and *groES*. The promoter region of *hrcA* was amplified using the primer pair *PhrcA_forw/PhrcA_rev* and that of *groES* was amplified with the primers *PgroES_forw/PgroES_rev*. EcoRI and Xhol were used to digest these DNA fragments and pSEUDO-GFP, in which the two promoter fragments were separately inserted. Insertion of the promoter-gfp fusions in the *llmg_pseudo_10* locus of the chromosome of *L. lactis* NZ9000 yielded the strains NZ9000::*PhrcA-gfp* and NZ9000::*PgroES-gfp*. By exposing both strains to high temperatures and making use of the stress-induced activity of *P_hrcA* and *P_groES*, it was demonstrated...
that they are able to reliably monitor, in a noninvasive manner (unlike, e.g., promoter-lacZ fusions) and in real time, the effect that they are able to reliably monitor, in a noninvasive manner.

Furthermore, the use of flow cytometry or other single-cell analysis techniques allows characterization of phenomena such as gene expression heterogeneity.

Altogether, pSEUDO, pSEUDO-GFP, and the derived plasmids and strains add value to the lactococcal research community in that they establish an improved working standard for the effective and efficient integration of DNA fragments into the chromosome of L. lactis.

REFERENCES