Construction of a plasmid vector based on the pMV158 replicon for cloning and inducible gene expression in *Streptococcus pneumoniae*

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Abstract

We report the construction of a plasmid vector designed for regulated gene expression in *Streptococcus pneumoniae*. The new vector, pLS1ROM, is based on the replicon of the streptococcal promiscuous rolling circle replication (RCR) plasmid pMV158. We inserted the controllable promoter P_M of the *S. pneumoniae malMP* operon, followed by a multi-cloning site sequence aimed to facilitate the insertion of target genes. The expression from P_M is negatively regulated by the transcriptional repressor MalR, which is released from the DNA operator sequence by growing the cells in maltose-containing media. To get a highly regulated expression of the target gene, MalR was provided in *cis* by inserting the *malR* gene under control of the constitutive P_{tet} promoter, which in pMV158 directs expression of the *tetL* gene. To test the functionality of the system, we cloned the reporter gene *gfp* from *Aequorea victoria*, encoding the green fluorescent protein (GFP). Pneumococcal cells harboring the recombinant plasmid rendered GFP fluorescence in a maltose-dependent mode with undetectable background levels in the absence of the inducer. The new vector, pLS1ROM, exhibits full structural and segregational stability and constitutes a valuable tool for genetic manipulation and regulated gene expression in *S. pneumoniae*.

1. Introduction

Streptococcus pneumoniae is a Gram-positive human pathogen that causes invasive infections such as pneumonia, meningitis or sepsis. In addition, pneumococcus is the leading bacterial cause of childhood mortality due to acute respiratory infections and nonepidemic meningitis in developing countries (WHO, 2009). Increasing antibiotic resistance of pathogenic *S. pneumoniae* is still an important problem despite the use of the conjugated pneumococcal vaccine and improvements in antimicrobial therapy (Perez-Trallero et al., 2010; Williams et al., 2002). Even though *S. pneumoniae* is a naturally transformable bacterium that is able to accept and transfer plasmids originally isolated from other species (Barany and Tomasz, 1980; Stassi et al., 1981), the occurrence of indigenous plasmids in pneumococcus seems to be very low. Most of the pneumococcal plasmids characterized so far are related to pDP1 (Smith and Guild, 1979). An exception is plasmid pSpnP1, which was identified in a multidrug-resistant strain of *S.*

pneumoniae (Romero et al., 2007). Sequence analysis showed that they are rolling-circle plasmids of the pC194/pUB110 family.

The availability of plasmid-based genetic tools to study the expression of native and heterologous genes in different microorganisms has a special interest for both basic research and practical applications. To date, the number of non-integrative plasmid vectors available to manipulate *S. pneumoniae* is still very poor. In fact, most of the constructs are based on the broad-host-range RCR plasmid pMV158, naturally isolated from *S. agalactiae* (Burdett, 1980), and on its derivative pLS1 (Stassi et al., 1981). In addition, the pneumococcal cryptic plasmid pRMG1 (related to pDP1) has been used to construct a shuttle vector between pneumococcus and *Escherichia coli* (Muñoz et al., 1999). Vectors based on the promiscuous pMV158 replicon have been used to clone pneumococcal genes and to analyze its heterologous expression (Espinosa et al., 1984; Lacks et al., 1986). Recently, a plasmid vector based on pLS1 has been presented as a useful genetic tool to study promoter and terminator sequences in *S. pneumoniae* and *Enterococcus faecalis* (Ruiz-Cruz et al., 2010). Plasmids pLS1RGFP (Nieto et al., 2000), pLS1GFP (Fernández de Palencia et al., 2000), pMV158GFP (Nieto and Espinosa, 2003) and pLS70GFP (Acebo et al., 2000) constitute another set of pMV158 replicon-based tools for the regulated expression of the *gfp* reporter gene. In these constructs, expression of *gfp* was controlled by the regulatory mechanism of maltosaccharides utilization in *S. pneumoniae* (Nieto et al., 1997). This system has been validated in pneumococcus (Nieto et al., 2000) and used to detect pneumococcal cells by fluorescence microscopy in culture medium (Nieto and Espinosa, 2003) or to confirm its intracellular location in granulocytes and microglia (Letiembre et al., 2005; Ribes et al., 2010).

In the present work we have constructed pLS1ROM, a new pLS1-based vector designed for cloning and regulated gene expression in *S. pneumoniae*. The presence of a multi-cloning site that helps the insertion of the target gene immediately after the maltose-inducible promoter P_M, the tight transcriptional control of P_M arising from the presence in *cis* of the MalR repressor (Nieto et al., 2000), and the minimization of the plasmid size by removal of dispensable regions are the more remarkable features of the pLS1ROM pneumococcal vector. The suitability of pLS1ROM as a regulated expression vector has been tested by cloning the *gfp* reporter gene and analyzing the fluorescence of pneumococcal cells containing the resulting

recombinant plasmid pLS1ROM-GFP upon maltose induction. In contrast to the previously constructed pLS1RGFP plasmid, pLS1ROM-GFP exhibits structural and segregational stability under induction conditions, which represents a significant improvement over the prior maltose-inducible pneumococcal system.

2. Materials and Methods

2.1. Bacterial strains and Plasmids

S. pneumoniae cells were grown in AGCH medium (Lacks, 1968) supplemented with 0.3% sucrose and 0.2% yeast extract, with selection for resistance to erythromycin (Ery, 1 µg/ml) when required. All cultures were grown at 37 °C. The constructs were done in *S. pneumoniae* 708 (*end-1 exo-1 trt-1 hex-4 malM594*; (Lacks and Greenberg, 1977). *S. pneumoniae* R6 (wild type; Lacks, 1968) was used for the expression of *gfp* and detection of GFP fluorescence. Other plasmids used were pLS1R (initially named as pAPM22; (Puyet et al., 1993), pCL1 (Nieto et al., 2000) and pGreenTIR (Miller and Lindow, 1997).

2.2. Plasmid DNA Preparation and DNA Manipulations

Pneumococcal cells were grown and transformed with plasmid DNA as previously reported (Lacks et al., 1986; Stassi et al., 1981). Plasmid DNAs purified from *S. pneumoniae* were prepared as described (del Solar et al., 1987). Restriction endonucleases, the Klenow fragment of DNA polymerase I (PolIK), and T4 DNA ligase were purchased from New England Biolabs or Roche, and were used according to the manufacturer's instructions. Restriction- or PCR-derived DNA fragments employed for cloning experiments were purified from agarose gels by phenol extraction as described (Sambrook et al., 1989). The duplex oligonucleotide MCS having 5'-protuding ends compatible with BamHI-generated ends was obtained by annealing complementary oligonucleotides MCSUP and MCSDOWN (MCSUP: 5'-GATCAAGCTTCCGCGGGCCCGGGCATGCGGCCGCACTAGTG-3'; MCSDOWN: 5'-GATCCACTAGTGCGGCCGCATGCCCGGGCCCGCGGAAGCTT-3'; protruding ends are shown underlined in both oligonucleotides), which were previously purified from gel as described (Maniatis, 1982). Reduction of the plasmid vector size was performed by inverse PCR (Ochman et al., 1988). Two different sets of divergent primers were employed to eliminate the

P_X promoter (DelX1: 5'-TCGATTCTGAGGCCTCTGAG-3'; DelX2: 5'-TACCTCCCTGGTTCTAATCC-3') and the *tetL* gene (Deltet1: 5'-GTCATTAGTTGGCTGGTTAC-3'; Deltet2: 5'-GGTTAATGATACGCTTCC-3'), by using pLS1ROXM-MCS and pLS1ROMtelL-MCS as template, respectively. After amplification with the Phusion polymerase (Finnzymes), the resultant DNA fragments were gel-purified and incubated with T4 DNA ligase to generate circular molecules that were used to transform *S. pneumoniae* cells. To allow ligation-sealing of the cyclized DNA molecules, the primers were phosphorylated at their 5' end previously to being used in the amplification reaction. All new plasmid constructs were confirmed by automated sequencing.

2.3. Determination of structural and segregational stability

Plasmid structural and segregational stability was determined in exponentially growing cultures of *S. pneumoniae* R6 containing either pLS1ROM or pLS1ROM-GFP. Pneumococcal cells were grown under different conditions (0.3% sucrose or maltose as carbon source; presence or absence of selective pressure (Ery 1 µg/ml) for the resident plasmid). Samples were taken after the indicated number of generations in order to analyze the integrity of the plasmid DNA (by electrophoresis of total DNA preparations in 1 % agarose gels; del Solar and Espinosa, 1992; Lacks et al., 1986), as well as the percentage of plasmid-containing (and hence Ery resistant) cells.

2.4. Measurement of GFP Fluorescence

To measure the GFP fluorescence inside the pneumococcal cells we followed the procedure described in (Nieto et al., 2000). Pneumococcal cells harboring pLS1ROM-GFP were grown in sucrose-containing media, with Ery resistance (Ery^R) selection, to an OD_{650} of 0.5 (about 4 x 10^8 c.f.u./ml) and then diluted 1:1000 into medium containing maltose, with or without selective pressure, and allowed to again reach an OD_{650} of 0.5. Cells (1 ml from each culture) were sedimented by centrifugation and suspended in the same volume of PBS buffer (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl), pH 7.2. Aliquots (200 µl) were used to measure the fluorescence intensity on a Varioskan Flash spectral scanning multimode reader (Thermo Scientific) by excitation at a wavelength of 488 nm and detection of emission at 510

nm. As a control to determine the background fluorescence, cells harboring the plasmid vector pLS1ROM (lacking the *gfp* gene) were used.

3. Results and Discussion

3.1. Construction of the regulated expression vector pLS1ROM

To construct the pLS1ROM vector we started with plasmid pLS1R, which has the pMV158 replicon and contains the *malR* gene cloned under the control of the P_{tet} promoter that directs constitutive expression of the tetracycline resistance *tetL* gene of pMV158 (Puyet et al., 1993) (Fig. 1). DNA from pLS1R was digested with HindIII and EcoRI, and treated with PolIK before purifying the larger fragment. To obtain a DNA region containing the operators/promoters P_X and P_M we used plasmid pCL1(Nieto et al., 2000). DNA from this plasmid was digested with SalI, treated with PolIK, and then digested with SspI. The resultant ~1 kb fragment was purified and ligated to the pLS1R larger fragment. The ligation mixture was used to transform *S. pneumoniae,* and transformants containing the resultant construction pLS1ROXM (Fig. 1) were selected for Ery^R. Transcription initiated from P_M is oriented in opposite direction to the other protein-encoding genes of pLS1ROXM (Fig. 1). Next step in the construction of the cloning vector was to introduce a multi-cloning site sequence (MCS) immediately after promoter P_M. To this end, the DNA from pLS1ROXM was linearized with BamHI and ligated to the 41-bp duplex oligonucleotide MCS, whose sequence had been designed to reconstitute a BamHI site at only one of its ends upon ligation with BamHI-generated fragments. Hence, the reconstituted BamHI site, which is to remain unique in the new construct pLS1ROXM-MCS, constitutes an additional single restriction site within the MCS. Moreover, the XbaI single site located between the P_M promoter and the MCS increases the number of restriction sites that can be used for cloning target genes (Fig. 1). Plasmid pLS1ROXM-MCS was obtained after transforming the ligation mixture into *S. pneumoniae* and selecting for Ery^R.

In order to improve the plasmid pLS1ROXM-MCS as an expression vector several modifications were carried out. First, we eliminated the $P_X/O_{X,}$ region, which is unnecessary for the MalR-mediated repression of P_M. In fact, the transcription rate from P_M has been reported to increase when the region encompassing P_X is deleted (Nieto et al., 2001). Region $P_X/O_{X,}$ was removed by inverse PCR, using the divergent phosphorylated primers DelX1 and DelX2, and

DNA from pLS1ROXM-MCS as template. The amplification reaction yielded a linear DNA fragment corresponding to almost the entire vector except the P_X/O_X region. The amplified fragment was gel-purified and subjected to auto-ligation to render circular plasmids molecules that were used to transform *S. pneumoniae*. The resultant plasmid was termed pLS1ROMtetL-MCS. Second, a ≈1.4 kb DNA fragment containing the *tetL* gene of pMV158 was eliminated. This gene was expressed neither in pLS1 nor in pLS1ROMtetL-MCS, as it was uncoupled from its promoter. To delete this sequence we followed the same strategy of inverse PCR but using the divergent phosphorylated primers Deltet1 and Deltet2, and DNA from pLS1ROMtetL-MCS as template. This deletion did not include the transcriptional terminator of the *tetL* gene, which was left to attenuate possible convergent transcription from the *ermAM* gene. The final plasmid vector pLS1ROM is 6.8 kb in size, confers Ery^R due to the presence of the *ermAM* gene, and facilitates the insertion of the target gene immediately after the regulated promoter P_M (GenBank accession JN381945).

3.2. Functionality of pLS1ROM: cloning and expression of the gfp gene in the regulated expression vector, and stability of the vector and recombinant plasmids

To assess the functionality of the constructed expression system, the *gfp* reporter gene from *A. victoria*, encoding GFP, was fused to the promoter P_M, and the fluorescence emission was measured. Specifically, we have used the *gfp* cassette from plasmid pGreenTIR, which was designed for prokaryotic transcriptional fusions and contains a mutated *gfp* gene (harboring the S65T “red shift” and F64L “protein solubility” amino acid changes), a translational enhancer and a consensus ribosome binding site, so that synthesis of GFP is enhanced. This optimized *gfp* cassette has been efficiently expressed in *S. pneumoniae* (Nieto et al., 2000) and in other Gram-positive hosts like *Bacillus subtilis* (Serrano-Heras et al., 2005), *Lactococcus lactis* (Fernández de Palencia et al., 2000), and *Enterococcus faecalis* (Lorenzo-Díaz and Espinosa, 2009; Ruiz-Cruz et al., 2010). Recombinant plasmid pLS1ROM-GFP was constructed by inserting the *gfp* cassette from pGreenTIR under the control of P_M. To this end, DNA from pLS1ROM was linearized with BamHI and the resultant fragment was purified. The *gfp* cassette was extracted by digesting DNA from pGreenTIR with BamHI. The resultant 810 bp DNA fragment was purified and ligated to the pLS1ROM fragment.

The stability of pLS1ROM and pLS1ROM–GFP was tested in cultures of *S. pneumoniae* R6 growing exponentially in media containing sucrose (repression conditions) and in the absence of Ery. The results showed that both plasmids were stably inherited, with no detectable appearance of Ery-sensitive plasmid-free cells over at least 100 generations (Fig. 2). Moreover, analysis of the total DNA content confirmed that the plasmids analyzed were stably maintained with no modification or rearrangement observed after 100 generations (Fig. 2).

Expression vectors and their recombinant derivatives harboring the gene to be expressed should ideally exhibit structural and segregational stability. It has been reported that RCR vectors for use in Gram-positive bacteria frequently become structurally and/or segregationally unstable upon insertion of even relatively small foreign DNA fragments (Grkovic et al., 2003; Leer et al., 1992). This is shown here not to be the case with pLS1ROM–GFP, which is stably maintained in pneumococcal cells, hence proving the suitability of pLS1ROM as a cloning vector for this bacterium.

We also analyzed the stability of the expression from the regulated promoter P_M by following the fluorescence emitted by the GFP protein in pLS1ROM-GFP-carrying pneumococcal cells that had been grown for several generations in the absence of both Ery and maltose. To induce GFP fluorescence, pneumococcal cultures grown under the above conditions for the indicated generations (Fig. 3A) were diluted 1:1000 into medium containing maltose and allowed to reach an OD_{650} of 0.5. The results showed that the fluorescence emission is maintained at similar levels in the interval of generations analyzed (Fig. 3A), indicating that the integrity and functionality of the transcriptional fusion between P_M and the *gfp* gene is preserved. These results are consistent with the new expression system being structurally and segregationally stable even in the absence of selective pressure, as demonstrated by the results shown in Figure 2.

Since transcription from the regulated promoter P_M converges on and might collide with that of the *ermAM* gene in both pLS1ROM (Fig. 1) and pLS1ROM-GFP (the cloned *gfp* gene lacked an intrinsic terminator), it was interesting to know whether, under induction conditions, expression of *ermAM* was affected so that selectable levels of Ery^R could not be achieved. To test this, we monitored the growth rate of pneumococcal cells carrying pLS1ROM or pLS1ROM-

GFP under induction conditions (maltose) with or without Ery^R selection. As a control, the same cells were grown in medium with sucrose (repression conditions), with or without Ery. The results (not shown) demonstrated that the duplication time of the pneumococcal cells grown in the presence of Ery was not significantly affected by the presence or absence of maltose, indicating that induction of transcription from P_M did not severely affect expression of the *ermAM* gene.

When analyzing the expression of *gfp* from the regulated promoter P_M, we observed that the fluorescence emitted in induced cultures of pneumococcal cells carrying pLS1ROM-GFP was remarkably higher than that of pneumococci harboring the previously-constructed plasmid pLS1RGFP (Fig. 3B). Furthermore, the cells carrying pLS1RGFP took approximately four times longer to duplicate in maltose-containing medium than in sucrose-containing medium, irrespective of the presence or absence of the antibiotic (not shown). Inhibition of the bacterial cell growth under induction conditions was accompanied by progressive plasmid loss, close to 100 % after 11 generations, in the absence of Ery, or by deletions reducing significantly the plasmid size in the presence of the antibiotic (Fig. 4). In contrast, the cells harboring pLS1ROM-GFP did not show any increase in their duplication time and the plasmid was stably maintained, at least during the generations analyzed, when grown in maltose-containing medium (Fig. 4). The instability of pLS1RGFP in induced pneumococcal cultures can explain the lower level of fluorescence observed in cells carrying this plasmid compared with those harboring pLS1ROM-GFP. The stability difference between these two pMV158 replicon-based plasmids may arise from promoter P_M directing transcription toward the replication origin in pLS1RGFP (Nieto et al., 2000), which could destabilize the plasmid under induction conditions. In contrast, pLS1ROM-GFP has P_M orientated in opposite direction relative to the plasmid replicon, and lacks the divergent P_X promoter.

4. Conclusions

In this manuscript we describe the construction of a new regulated expression vector, pLS1ROM, which is based on the streptococcal pMV158 replicon and has been specially designed to clone and express both native and heterologous genes in *S. pneumoniae*. We have included a MCS sequence to facilitate the insertion of the target gene, improved the expression

from the regulated promoter P_M and reduced the plasmid size to increase the stability of the vector. We have proved the suitability of this vector by cloning and willfully expressing the *gfp* gene. In conclusion, pLS1ROM is a stable and functional expression vector that increases the spectrum of genetic tools available to manipulate *S. pneumoniae*.

Acknowledgments

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Figure Legends

Figure 1. Regulated expression vector pLS1ROM. (A) Construction of pLS1ROM. The various steps in the construction of pLS1ROM are indicated. PMV158-born genes (black) and the *ermAM* gene (grey) are indicated by arrows. Chromosomal-born *malR* gene and the region containing the promoters P_M and P_X are indicated by open arrows and open boxes respectively. The promoter sequences are indicated by triangles pointing in the direction of transcription. (B) Sequence of the plasmid vector region encompassing the inducible promoter P_M, the operator O_M and the MCS.

Figure 2. Structural and segregational stability of pLS1ROM and pLS1ROM-GFP. Total DNA extracted from cultures of pLS1ROM- or pLS1ROM-GFP-harboring pneumococcal cells that had been grown for the indicated number of generations in medium lacking Ery was analyzed by 1% agarose gel electrophoresis (left panel). To facilitate the identification of the main plasmid forms, pLS1ROM and pLS1ROM-GFP DNA samples were obtained by the alkaline lysis method and loaded to the left of the corresponding total DNA extracts. OC, open circular DNA; SC, supercoiled monomeric plasmid DNA. The percentage of Ery-resistant colonies is given in the right panel.

Figure 3. GFP fluorescence in pneumococcal cells harboring pLS1ROM-GFP. (A) Pneumococcal cells carrying pLS1ROM or pLS1ROM-GFP were grown in medium lacking both Ery and maltose for the indicated number of generations. Production of GFP was subsequently induced by growing these cells for 10 generations in the presence of maltose. Fluorescence background levels were obtained from cells grown in the absence of maltose for 10 more generations. (B) The fluorescence emitted by pneumococcal cells carrying pLS1ROM-GFP under induction (maltose) and repression (sucrose) conditions was compared with the fluorescence due to the previously reported construction pLS1RGFP (Nieto et al., 2000) under the same conditions. The fluorescence emission data of pneumococcal cells carrying the *gfp*-less plasmids pLS1R and pLS1ROM were included as a control.

Figure 4. Structural and segregational stability of pLS1RGFP, pLS1ROM and pLS1ROM-GFP under induction conditions. Pneumococcal cultures harboring the indicated plasmids were grown to an OD_{650} of 0.5 in medium containing sucrose with Ery^R selection (generation 0), and

then diluted 1:2000 into medium containing maltose, with or without Ery. After growing the cultures for the indicated number of generations total DNA preparations were analyzed by 1% agarose gel electrophoresis. As a control the same pneumococcal cultures were diluted into medium containing sucrose, with or without Ery, and subjected to the same analysis. The main plasmid forms of pLS1ROM and pLS1ROM-GFP were identified in DNA samples prepared by alkaline lysis (lanes C). OC, open circular DNA; SC, supercoiled monomeric plasmid DNA; Del, deleted plasmid DNA.

Figure 1

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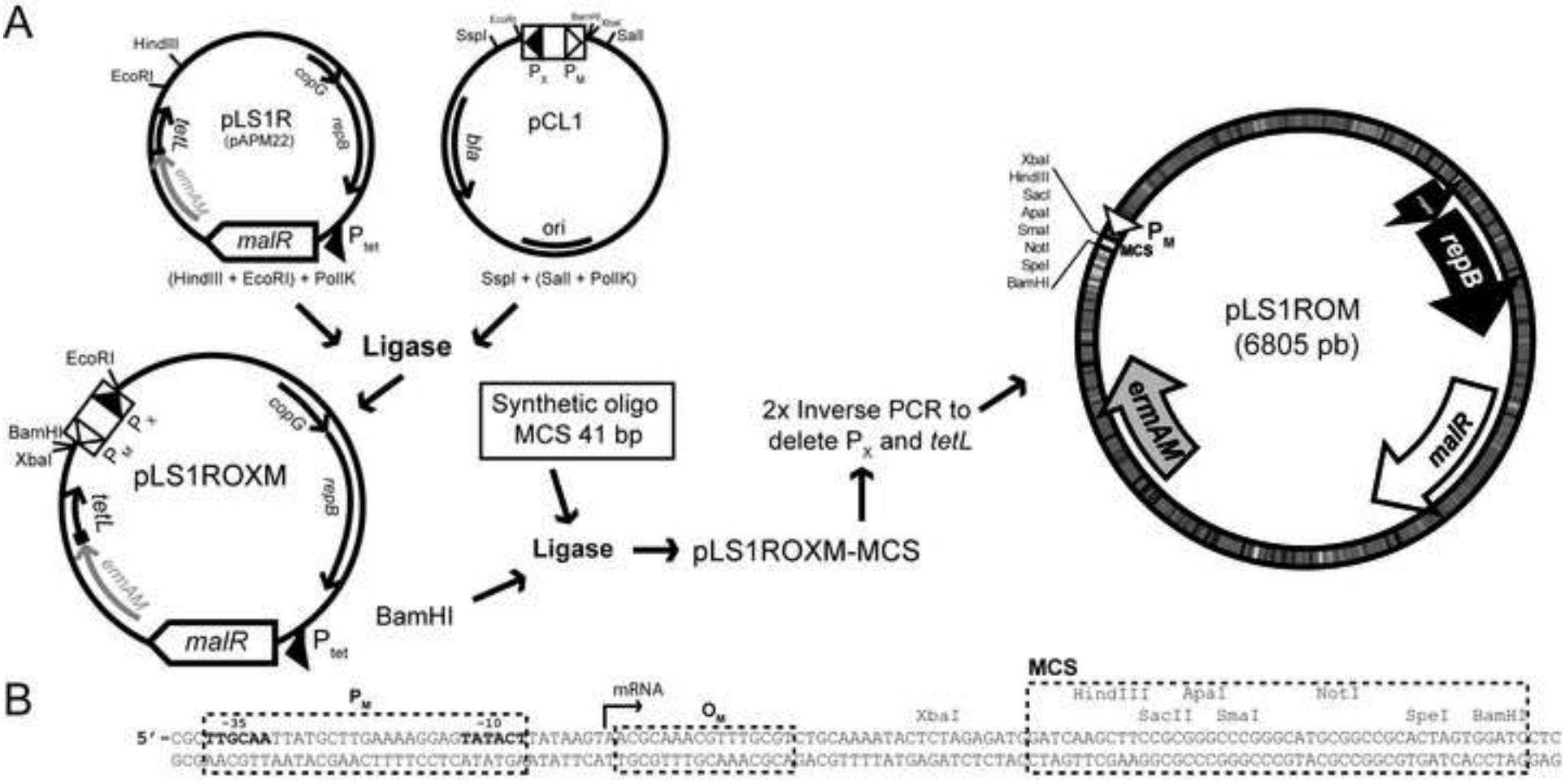

Figure 2

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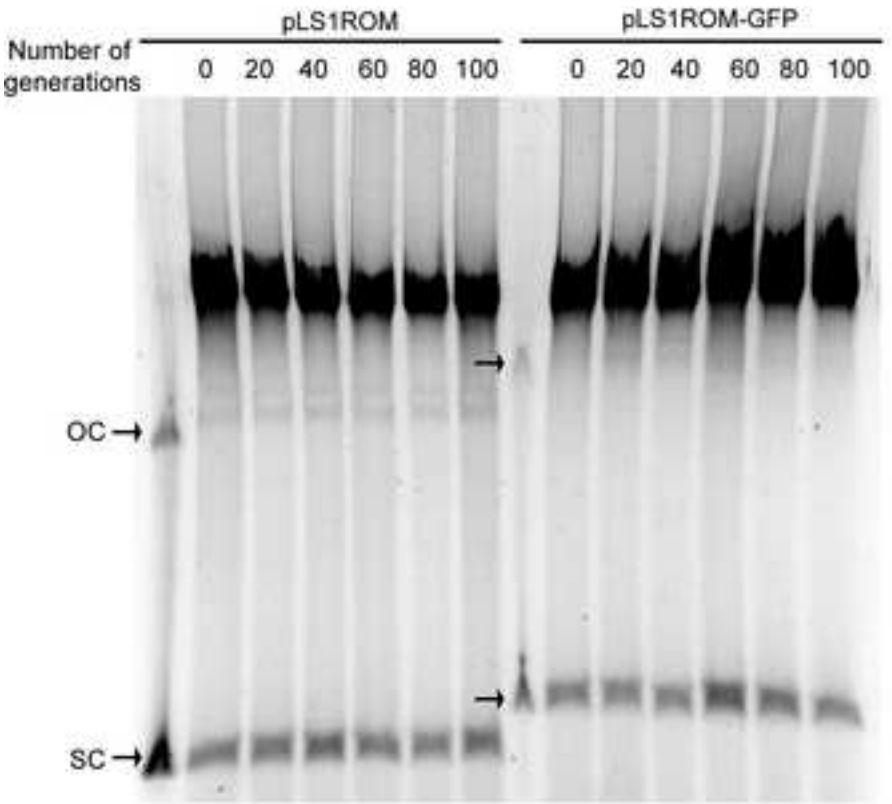

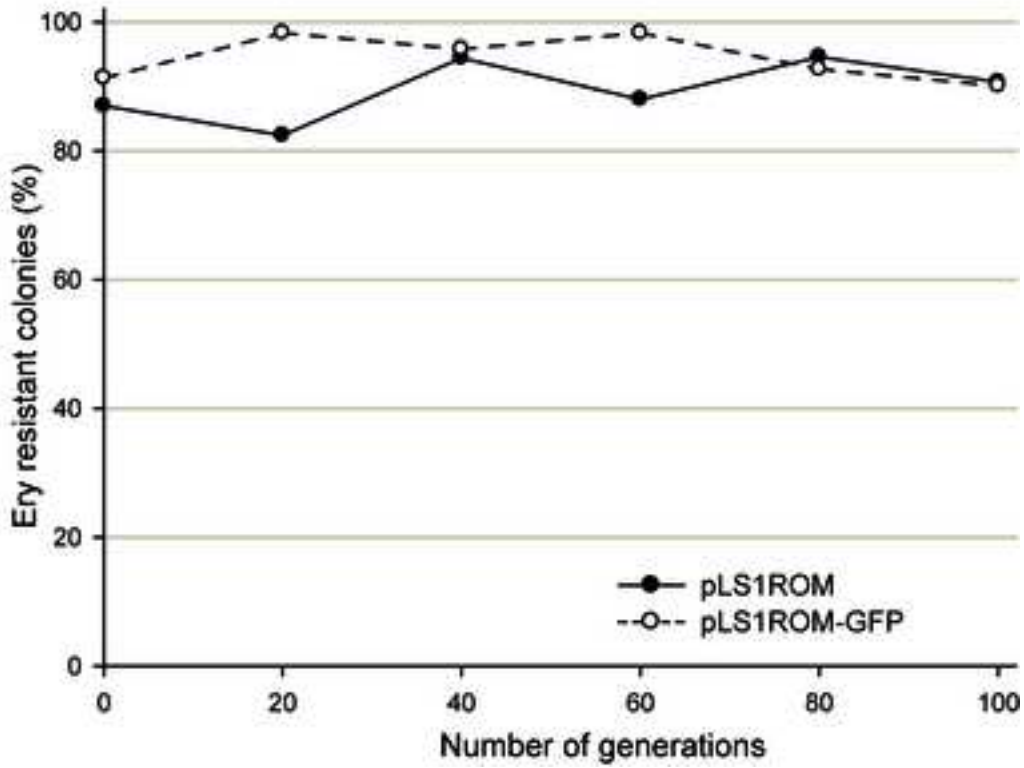

Figure 3

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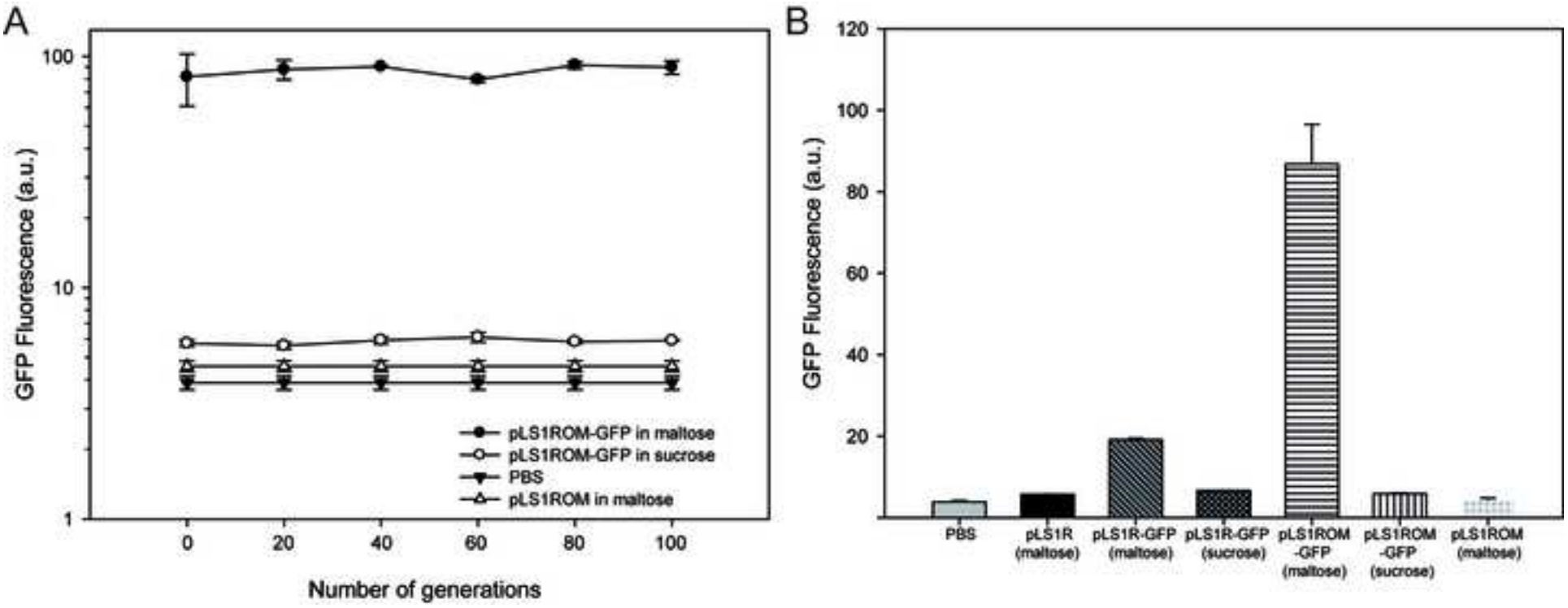

Figure 4

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