A new tool for cloning and gene expression in *Streptococcus pneumoniae*

Susana Domingues[a,*, ✉], Andreia Cunha Aires[a,*], Mari Luz Mohedano[b], Paloma López[b] and Cecília M. Arraiano[a]

[a] Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, 2780-157 Oeiras, Portugal.

[b] Departamento de Microbiología Molecular y Biología de las Infecciones, Centro de Investigaciones Biológicas CIB (CSIC), 28040 Madrid, Spain.

[*] These authors contributed equally to this work.

Keywords: pneumococcal cloning, expression vector, replicating plasmid, non-integrative vector

✉Corresponding author: Susana Domingues
Tel: +351 214469560
Fax: +351 214469549
E-mail: susanadomingues@itqb.unl.pt

Abstract

A new replicon suitable for cloning and gene expression was successfully introduced into *Streptococcus pneumoniae*. The non-integrative lactococcal vectors pIL253 (high-copy) and pIL252 (low-copy), which are based on the promiscuous theta-replicating plasmid pAMβ1, were established in pneumococcus. The stability and the small size of these plasmids, together with the presence of a helpful multi-cloning site make them a useful genetic tool for gene expression in this bacterium. The functionality of the system was tested by cloning and expressing the pneumococcal RNase R in pIL253. Full constitutive expression of the cloned gene was observed, clearly demonstrating that this plasmid can be used as an expression vector in *S. pneumoniae*. Moreover, gene expression can be regulated by the use of the low- or high-copy vector versions. The existence of other replicative plasmids based on this family, which are also probably functional in pneumococcus, further broadens the cloning possibilities. We also show that *S. pneumoniae* cells can accommodate simultaneously pIL252 or pIL253 together with pLS1plasmid, a pMV158 derivative, which replicates *via* rolling circle mechanism. This fact greatly increases the ability to manipulate this bacterium.

The availability of a new family of replicative vectors for genetic manipulation in *S. pneumoniae* is an important contribution to the study of this pathogenic microorganism.

1. Introduction

The pathogenic Gram-positive bacterium *S. pneumoniae* is one of the leading causes of nosocomial infections. Although normally found as a harmless commensal of the human upper respiratory tract, it may cause invasive infections like pneumonia, meningitis or sepsis, when the immune system weakens. Normal disease development begins with colonization of the nasopharynx followed by progression to the lung or bloodstream. Collectively, these diseases result in millions of deaths worldwide each year, affecting especially young children and the elderly. Understanding the mechanisms of pathogenesis is essential to identify new potential targets that can be used to design innovative drugs effective to combat this microorganism. This has prompted increased efforts to study this bacterium at the molecular level, which often requires the use of refined genetic tools. Although *S. pneumoniae* is a naturally transformable bacterium only a few indigenous cryptic plasmids have been identified and most of the plasmid vectors used for its manipulation were isolated from heterologous

hosts. A shuttle vector between *S. pneumoniae* and *Escherichia coli* based on an indigenous plasmid has also been constructed {Munoz, 1999 #12}. However, non-integrative plasmid vectors are still very scarce and most of the studies have been carried out on the broad host range, rolling circle replicating (RCR) plasmid pMV158 {Burdett, 1980 #13} and on its derivative, pLS1 {Stassi, 1981 #14}. A set of plasmids based on this promiscuous replicon that can be used as *gfp*-fusions vectors using Green Fluorescent Protein (GFP) as a reporter for gene expression have been constructed {Acebo, 2000 #17;Fernandez de Palencia, 2000 #15;Nieto, 2000 #16;Nieto, 2003 #18}. An improved vector for regulated gene expression based on the same system has recently been reported {Ruiz-Maso, 2012 #19}. pLS1 derivatives allowing expression of mCherry or citrine fusion proteins have also been designed {Henriques, 2011 #20} and a plasmid tool based on pLS1 is available to study promoter and terminator sequences in *S. pneumoniae* and *Enterococcus faecalis* {Ruiz-Cruz, 2010 #21}. However, most of the pLS1 derivatives are large plasmids that are not easy to handle. Most of them lack a useful multi-cloning site and the respective sequence is often not available.

These observations prompted us to search for cloning vectors based in another replicon that could be suitable for the expression of genes in *S. pneumoniae*. pIL253 is a pAMβ1-based plasmid, a well characterized theta-replicating promiscuous replicon isolated from *Enterococcus faecalis* {Bruand, 1991 #22}. Plasmids belonging to the pIL253 family were initially constructed for molecular cloning in *Lactococcus lactis* and have been extensively used for gene cloning and expression in Gram-positive bacteria. This is mainly due to their small size (< 5 kb) and stability, the presence of a useful multi-cloning site (MCS), sequence availability and their demonstrated ability to stably maintain large DNA inserts in *L. lactis* {Simon, 1988 #7}. Furthermore they are available in low- (pIL252) or high- (pIL253) copy number versions {Simon, 1988 #7}, which allows a better control of gene expression.

In this work we have examined the capability of these plasmids to be used as vectors for cloning and gene expression in *S. pneumoniae*. We show that pIL253 autonomously replicates and is stably maintained in this bacterium. Cloning of RNase R into pIL253 yielded high levels of RNA and protein, readily demonstrating the utility of this plasmid family as a pneumococcal expression vector. In addition the compatibility of pIL252 or pIL253 with pLS1, demonstrated in this work, is undoubtedly one the most useful properties of these plasmids.

2. Material and methods

2.1. Bacterial strains and growth conditions

All strains used in this work are listed in Table 1. *S. pneumoniae* TIGR4 {Tettelin, 2001 #1} and derivatives were routinely grown in Todd Hewitt medium, supplemented with 0.5 % yeast extract (THY), at 37°C without shaking or in Trypticasein soy broth (TSB) agar plates supplemented with 5% sheep blood. When required the growth medium was supplemented with 2.5 or 3 µg/ml chloramphenicol (Cm), 5 µg/ml erythromycin (Ery) or 1 µg/ml tetracycline (Tet) as specified bellow. *E. coli* was cultivated in Lysogeny broth (LB) at 37 ºC with agitation. The growth medium was supplemented with 100 µg/ml ampicillin (Amp) when required.
All oligonucleotides used in this work were synthesized by STAB Vida, Portugal.

2.2. DNA manipulations and plasmids

The lactococcal plasmid vectors pIL252 and pIL253 {Simon, 1988 #7} were transformed into *S. pneumoniae* TIGR4. Pneumococcal transformation was performed essentially as described {Burghout, 2007 #2}. Briefly pre-competent pneumococcal cells were grown for 10 min at 37°C in a 10-fold volume of CTM-pH 7.8 {Burghout, 2007 #2} supplemented with 125 ng/ml CSP-2 (the synthetic competence-stimulating peptide 2). Media were supplemented with Tet, when cells previously carried pLS1. 200 ng of plasmid DNA were then added and cultures were further incubated at 30 ºC for 30 min and then shifted to 37 ºC. One hour and thirty minutes after the temperature shift, transformants were selected on solid medium supplemented with 1 µg/ml Ery.
Isolation of plasmid DNA from *S. pneumoniae* was performed using the Wizard Plus SV Minipreps DNA purification system (Promega) with some modifications. Exponentially growing cells were harvested and resuspended in TES (25 mM Tris pH 8, 10 mM EDTA, 1.7 % saccharose) supplemented with 8 mg/ml lysozyme and incubated at 37 ºC for 15 min. The suspension was centrifuged at 2350 x g for 5 min, and the cell pellet was resuspended in 0.9 M NaCl (200 µl) and centrifuged again under the same conditions. The pellet was then processed following the supplier's instructions. Plasmid DNA was digested with PstI (Fermentas), as recommended by the supplier.
pIL253 carrying the coding sequence of the TIGR4 RNase R inserted into the SmaI/PstI sites of the plasmid MCS {Moreira, in press #29} was used to assess the ability of pIL253 to stably

carry and express a DNA fragment in *S. pneumoniae*. Expression of RNase R was evaluated by transforming this construction into a *S. pneumoniae* TIGR4 RNase R^- mutant strain (see below). Transformants were selected with 1 μg/ml Ery and were further propagated in media containing Cm (3 μg/ml) and Ery (1 μg/ml).

The *S. pneumoniae* RNase R deficient mutant was created through allelic replacement mutagenesis, using a DNA fragment containing the *rnr* flanking regions, in which *rnr* is replaced by a chloramphenicol resistance cassette (*cat*). The *cat* marker was amplified with primers smd013 (GATAAGCTTGATGAAAATTTG) and smd014 (CATTATCTCATATTATAAAAG). The upstream and downstream *rnr* flanking regions were amplified by PCR using the primer pairs smd011 (5'-CCGGAATTCGGACTCTATCGTTTCCTCTTTG-3') / smd015 (5'-CAAATTTTCATCAAGCTTATCATTTTCTTTCTAGATTTTTTAATTTTC-3') and smd016 (5'-CTTTTATAATATGAGATAATGAAGGAAGTAGCTAAGAAAGGAG-3') / smd012 (5'-CGCGGATCCAATGAACAGGATTGGTATTG-3'), respectively. Primers smd015 and smd016 included 3' extensions complementary to the 5'- and 3'-end respectively of the *cat* marker. The PCR amplified fragments with the complementary extensions were used as templates together with the *cat* marker to obtain a final PCR product. This product, which contained the *rnr* flanking regions surrounding the *cat* cassette, was inserted into the sites EcoRI/BamHI of pUC19 (Invitrogen) and propagated in *E. coli* DH5α. The recombinant plasmid was purified as described by *Birnboim and Doly* {Birnboim, 1979 #27}, linearized with EcoRI and used to transform *S. pneumoniae* 708 strain {Lacks, 1970 #28}. Competence and transformation procedures were performed according to {Lacks, 1968 #26}. Transformants were selected with Cm (2.5 μg/ml), and the correct nucleotide sequence of the *rnr::cat* chromosomal insert was confirmed by DNA sequencing. Then, chromosomal DNA from *S. pneumoniae* 708 (*rnr*::*cat*) mutant (1 μg/ml*)* and the synthetic CSP-2 (25 ng/ml) were used to transform the *S. pneumoniae* TIGR4 strain as above. Transformants containing the *rnr*::*cat* in their chromosome were selected for Cm (2.5 μg/ml) and the correct nucleotide sequence was confirmed by DNA sequencing.

2.3. Plasmid segregational stability

Segregational stability was tested according to Simon and Chopin {Simon, 1988 #7}. Briefly, an overnight culture of *S. pneumoniae* TIGR4 containing pIL253 grown in medium supplemented with Ery was diluted to an OD_{600} of 0.05 in fresh THY without antibiotic. The culture was allowed to grow until early stationary phase and then plated onto non-selective TSB agar. The fraction of erythromycin-resistant clones was determined by transferring ~150 of those colonies onto erythromycin-free and erythromycin-containing TSB agar plates.

2.4. Determination of copy number

Pneumococcal cultures of TIGR4 carrying pLS1, pIL252 or pIL253, were grown to an OD_{650} = 0.45. Total DNA was prepared and fractionated in 1% agarose as described by Acebo *et al.* {Acebo, 1996 #30}. Gels were stained with ethidium bromide (0.5 µg mL^{-1}) for 1 h and the intensity of the DNA bands was quantified by use of a Gel Doc 2000 instrument and the Quantity One 4.6.5 software (BioRad laboratories)

The average number of plasmid copy number (from four determinations) was calculated by the use of the following equation developed by Projan *et al*. {Projan, 1983 #31}:

$$N = \frac{(Dp1 + 1.36\ Dp2)x\ Mc}{Dc\ x\ Mp}$$

Where Dp_1 and Dp_2 are, respectively, the intensity values determined for the open and covalently closed plasmid forms. M_c is the molecular weight of the *S. pneumoniae* TIGR4 chromosome (2160837 bp) and D_c is the intensity value for the chromosomal DNA. Mp is the molecular weight of the plasmids: pLS1 (4408 bp), pIL252 (4698 bp) and pIL253 (4963 nt). The coefficient 1.36 is introduced to correct the differences in fluorescence due to the efficiency of ethidium bromide for intercalation in open circle and supercoiled DNA.

2.5. RNA Extraction

Overnight cultures of *S. pneumoniae* TIGR4 wild type and derivatives were diluted in pre-warmed THY to a final OD_{600} of 0.1, and incubated at 37 ºC until OD_{600} ~ 0.3. At this point, cultures were split into two aliquots and each was further incubated at 15 ºC or 37 ºC for 2 h. 20 ml culture samples were collected, mixed with 1 volume of stop solution (10 mM Tris pH 7.2, 25 mM $NaNO_3$, 5 mM $MgCl_2$, 500 µg/ml chloramphenicol) and harvested by

centrifugation (10 min, 2800 x g, 4 ºC). Total RNA was extracted using Trizol reagent (Ambion) essentially as described by the manufacturer, with some modifications. Pneumococcal cells were lysed by incubation in 650 µl lysis buffer (sodium citrate 150 mM, saccharose 25 %, sodium deoxicholate 0.1 %, SDS 0.01 %) for 15 min at 37 ºC followed by addition of 0.1 % SDS. After lysis, samples were treated with 10 U Turbo DNase (Ambion) for 1 h at 37 ºC. After extraction, the RNA integrity was evaluated by gel electrophoresis and its concentration estimated using a Nanodrop 1000 machine (Nanodrop Technologies).

2.6. Reverse Transcription-PCR (RT-PCR)

RT-PCR reactions were carried out using total RNA, with the OneStep RT-PCR kit (Qiagen), according to the supplier's instructions. The primer pair seqT4-2 (GACATCGCTATAGGTCATACG) / seqT4-3(GTTTGACAACAGTTGTCGGG) was used to analyze *rnr* expression. As an independent control, 16S rRNA was amplified with specific primers 16sF (AGAGTTTGATCCTGGCTCAG) and 16sR (ACGGCTACCTTGTTACGACTT). Prior to RT-PCR, all RNA samples were treated with Turbo DNA free Kit (Ambion). Control experiments, run in the absence of reverse transcriptase, yielded no product.

2.7. Total Protein Extraction and Western Blotting

Cell cultures used to prepare protein extracts were grown in the same conditions as described above for RNA extraction. 20 ml culture samples were collected, mixed with 1 volume of stop solution (defined above) and harvested by centrifugation (10 min, 2800 xg, 4 ºC). The cell pellet was resuspended in 100 µl of TE buffer supplemented with 1 mM PMSF, 0.15 % sodium deoxycholate and 0.01 % SDS. After 15 min incubation at 37 ºC, SDS was added to a final concentration of 1 %. Protein concentration was determined using a Nanodrop 1000 machine (NanoDrop Technologies). 20 µg of total protein were separated in a 7 % tricine-SDS-polyacrylamide gel, following the modifications described by {Haider, 2010 #3}. After electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare) by electroblotting using the Trans-Blot SD semidry electrophoretic system (Bio-Rad). The membrane was then probed with 1:500 dilution of anti-RNase R antibodies {Moreira, in press #29}. ECL anti-rabbit IgG peroxidase conjugated (Sigma) was used as the secondary antibody in a 1:10000 dilution. Immunodetection was conducted via a chemiluminescence reaction using Western Lightning Plus-ECL Reagents (PerkinElmer).

3. Results and discussion

3.1. pIL253 autonomously replicates in S. pneumoniae

The scarcity of non-integrative plasmid-vectors available to genetically manipulate *S. pneumoniae* (and which are mainly based on the replicon pMV158), associated with difficulties in handling the existent tools, prompted us to examine the ability of the lactococcal plasmid-vector pIL253 to autonomously replicate in *S. pneumoniae*.

For this purpose, pIL253 was transformed into *S. pneumoniae* TIGR4 and Ery resistant clones were analyzed for the presence of the plasmid. As shown in Fig. 1 pIL253 was isolated from exponentially growing transformants propagated in selective medium, indicating that this plasmid is able to autonomously replicate in pneumococcus. However, although pIL253 is stable in *S. pneumoniae* in the presence of selective pressure, only 5 % of the cells still carried the plasmid after continuous growth in the absence of Ery. The plasmid extracted from these cells showed the same electrophoretic mobility forms as observed in the original *L. lactis* preparation. One fragment corresponding to the size of pIL253 (~5 kb) was observed after linearization of both plasmid preparations with the single cutter PstI (Fig. 1, lanes 3-4). Thus the plasmid isolated from *S. pneumonia* seems to be identical to the original *L. lactis* plasmid. The overall results indicate that pIL253 is structurally stable in *S. pneumoniae* and it can be stably maintained under selective pressure

Since the use of low-copy number vectors is often an advantage, we also tested pIL252, which is a low-copy number version of pIL253. The *rep* promoter activity is repressed by the product of the *repF* gene, which is present in pIL252 but is disrupted in pIL253, leading to the respective low and high copy numbers of these vectors {Simon, 1988 #7}. Analogously to pIL253, pIL252 can only be stably maintained in S. pneumoniae under selective pressure: in the absence of the antibiotic only 5 % of the cells retain the plasmid.

Together, the results presented here show that this vector family can be propagated in pneumococcal cells under antibiotic selective pressure.

3.2. pIL252/pIL253 compatibility with pLS1

An important property of a cloning vector is its compatibility with other vectors. Because they use different replication mechanisms, RCR- and theta-replicating vectors are often

compatible. Thus we tested if pIL253 could be maintained in *S. pneumoniae* together with pLS1.

To this purpose *S. pneumoniae* TIGR4 cells carrying pLS1 were transformed with pIL252 or pIL253 and transformants, selected in media containing Tet and Ery, were tested for the presence of both plasmids. Plasmids extracted from exponentially growing single transformants were then compared with those extracted from double transformants by restriction analysis. The enzyme chosen (PstI) is a single cutter for pIL252/pIL253 and a double cutter for pLS1, giving rise to different sized fragments that can be easily identified. The result obtained with pIL252 is shown in Fig. 2. An analogous result was obtained when pIL253 was used instead (data not shown). The restriction analysis of plasmid DNA indicates that none of the plasmids underwent structural rearrangements. Thereby this result shows that pneumococcal cells are able to stably maintain both pIL252/pIL253 vectors and pLS1. This feature represents a major advantage for their genetic manipulation.

3.3 Determination of pIL252 and pIL253 copy number

Analysis of total DNA preparations (Fig. 3) revealed that pIL253 as well as pLS1 are mainly present in monomeric supercoiled forms, whereas in some instances pIL252 multimers where observed in *S. pneumoniae* TIGR4.

Determination of plasmid copy number revealed the expected value of 20 $\pm$ 2 for pLS1 as previously described {Lacks, 1986 #32;Acebo, 1996 #30}. The pIL252 plasmid presented a copy number of 27 $\pm$ 2, whereas pIL253 showed 59 $\pm$ 5. In addition, supporting the compatibility of pLS1 and the pIL plasmids, no significant influence in the copy number was observed by carrying pLS1/pIL252 or pLS1/pIL253 plasmids. However, unexpectively, it seems that in the presence of pLS1, pIL252 decreases the degree of multimerization.

3.4. Gene cloning and expression from pIL253

To assess the functionality of this plasmid as an expression vector in pneumococcus, the coding sequence of *S. pneumoniae* TIGR4 RNase R was inserted into the pIL253 multi-cloning site and this construction was transferred into an RNase R deficient strain. RNase R expression from pIL253 was then evaluated by RT-PCR and Western blotting. Since RNase R is known to be a protein that exhibits the highest expression levels under cold shock {Cairrão, 2003 #4;Moreira, in press #29}, the experiments were conducted at 15 ºC and 37

ºC, to have a better comparison with RNase R expression from the chromosome. The results in Fig. 4 show that RNase R is highly expressed from pIL253. Both mRNA and protein levels are higher than those detected with the wild type strain even at 15 ºC. We also observe that expression from this plasmid seems to be constitutive and does not change with temperature. This plasmid has been extensively used for expression of homologous and heterologous genes in lactic acid bacteria and high expression levels have previously been reported (see for instance {de Vos, 1990 #8;van Alen-Boerrigter, 1991 #6;Kleerebezem, 1997 #9;Domingues, 2004 #10}. The high expression level may be a consequence of the gene dosage effect, since the copy number of pIL253 was calculated to be about 57 copies / chromosome. Moreover, this vector is known to contain a promoter in the replication region that reads through into the multiple cloning site {de Vos, 1990 #8} and it is probably implicated in the expression of genes cloned in the same transcriptional orientation. Full detection of RNase R in late exponential cultures indicates that the DNA insert remains stable after several generations. In *L. lactis* this vector is able to stably support large DNA fragments (up to 30 kb) {Simon, 1988 #7}. Theta-replicating plasmids are in general structurally more stable than RCR plasmids and, for this reason they can accommodate and maintain large inserts {Kiewiet, 1993 #5}. Taken together these results clearly demonstrate that the lactococcal vector pIL253 is suitable for gene cloning and expression in *S. pneumoniae*.

4. Conclusions

A replicon derived from the stable theta-replicating plasmid pAMβ1, was introduced for the first time in *S. pneumoniae*. The pIL253 vector, a pAMβ1 derivative, was successfully used for the cloning and expression of a pneumococcal chromosomal gene in host cells of this species. Moreover, this small sized vector contains a functional multi-cloning site with several unique restriction sites and it is able to stably maintain large DNA inserts. The possibility of using the low-copy number version (pIL252) offers another option for the genetic manipulation of pneumococcus. Moreover, the availability of other plasmid refined tools constructed on the basis of this family with different cloning possibilities constitutes an extra advantage. Besides pIL253 and pIL252, pIL871, a chloramphenicol resistance derivative of pIL253 was also constructed {Domingues, 2004 #10}. pIL253 and pIL871 share the same replication protein and can be stably maintained in a mixture by double selection with erythromycin and

chloramphenicol in *L. lactis* {Domingues, 2004 #10}. This, which would allow the simultaneous expression of genes cloned independently in each of the vectors, is also an open possibility that can be tested in *S. pneumoniae*. We have also shown that pIL252/pIL253 can be maintained in *S. pneumonia* simultaneously with pLS1. This result indicates that these replicons are compatible, which significantly broadens the applications of these cloning vectors.

An interesting collection of plasmids (pTRK family) offers low- and high-copy number shuttle vectors carrying the *E. coli* p15A origin of replication incorporated respectively into pIL252 and pIL253 {O'Sullivan, 1993 #23}. Some of these vectors enable blue/white screening of clones in *lacZα*-complementing *E. coli* strains. Moreover these vectors have been successfully introduced into *Lactococcus*, *Enterococcus*, *Lactobacillus* and *Streptococcus* {O'Sullivan, 1993 #23}, which reinforces the possibility to extend their use for the genetic manipulation of *S. pneumoniae*. The pIA plasmid family also supplies a collection of shuttle vectors that include pIL253-based plasmids. Extended multi-cloning sites were added together with different selective markers and the possibility of X-gal selection of recombinant colonies {Perez-Arellano, 2001 #24}. This is also a broad host-range plasmid family that has been introduced in several Gram-positive bacteria and may thus also be suitable for gene cloning into *S. pneumoniae*.

We believe that this work, by introducing the use of novel plasmid-based refined genetic tools, opens new perspectives for the study and genetic manipulation of the important human pathogen *S. pneumoniae*.

Acknowledgments

We acknowledge Alain Chopin for the kind supply of pIL253 and pIL252.

Fundação para a Ciência e Tecnologia gave financial support to S. Domingues (Postdoctoral fellowship) and A. C. Aires (Fellowship). This work was supported by *Fundação para a Ciência e a Tecnologia* including grant # PEst-OE/EQB/LA0004/2011 and the Spanish Ministry of Economy and Competitiveness grant AGL2012-40084-C03-01.

References

Acebo, P., et al., 2000. Quantitative detection of *Streptococcus pneumoniae* cells harbouring single or multiple copies of the gene encoding the green fluorescent protein. Microbiology. 146 (Pt 6)**,** 1267-73.

Birnboim, H. C., Doly, J., 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7**,** 1513-23.

Bruand, C., et al., 1991. Unidirectional theta replication of the structurally stable *Enterococcus faecalis* plasmid pAM beta 1. Embo J. 10**,** 2171-7.

Burdett, V., 1980. Identification of tetracycline-resistant R-plasmids in *Streptococcus agalactiae* (group B). Antimicrob Agents Chemother. 18**,** 753-60.

Burghout, P., et al., 2007. Search for genes essential for pneumococcal transformation: the RADA DNA repair protein plays a role in genomic recombination of donor DNA. J Bacteriol. 189**,** 6540-50.

Cairrão, F., et al., 2003. Cold shock induction of RNase R and its role in the maturation of the quality control mediator SsrA/tmRNA. Mol. Microbiol. 50**,** 1349-1360.

de Vos, W. M., et al., 1990. Characterization of the lactose-specific enzymes of the phosphotransferase system in *Lactococcus lactis*. J Biol Chem. 265**,** 22554-60.

Domingues, S., et al., 2004. The Lactococcal abortive phage infection system AbiP prevents both phage DNA replication and temporal transcription switch. J Bacteriol. 186**,** 713-21.

Fernandez de Palencia, P., et al., 2000. Expression of green fluorescent protein in *Lactococcus lactis*. FEMS Microbiol Lett. 183**,** 229-34.

Haider, S. R., et al., 2010. Modification of tricine-SDS-PAGE for online and offline analysis of phosphoproteins by ICP-MS. Anal Bioanal Chem. 397**,** 655-64.

Henriques, M. X., et al., 2011. Synthesis of capsular polysaccharide at the division septum of *Streptococcus pneumoniae* is dependent on a bacterial tyrosine kinase. Mol Microbiol. 82**,** 515-34.

Kiewiet, R., et al., 1993. The Mode of Replication Is a Major Factor in Segregational Plasmid Instability in *Lactococcus lactis*. Appl Environ Microbiol. 59**,** 358-64.

Kleerebezem, M., et al., 1997. Controlled gene expression systems for lactic acid bacteria: transferable nisin-inducible expression cassettes for *Lactococcus*, *Leuconostoc*, and *Lactobacillus* spp. Appl Environ Microbiol. 63**,** 4581-4.

Lacks, S., 1968. Genetic regulation of maltosaccharide utilization in Pneumococcus. Genetics. 60**,** 685-706.

Lacks, S., 1970. Mutants of *Diplococcus pneumoniae* that lack deoxyribonucleases and other activities possibly pertinent to genetic transformation. J Bacteriol. 101**,** 373-83.

Moreira, R. N., et al., 2012. Synergies between RNA degradation and *trans*-translation in *Streptococcus pneumoniae*: cross regulation and co-transcription of RNase R and SmpB BMC Microbiology, *in press*.

Munoz, R., et al., 1999. Construction of a new *Streptococcus pneumoniae-Escherichia coli* shuttle vector based on the replicon of an indigenous pneumococcal cryptic plasmid. Int Microbiol. 2**,** 23-8.

Nieto, C., Espinosa, M., 2003. Construction of the mobilizable plasmid pMV158GFP, a derivative of pMV158 that carries the gene encoding the green fluorescent protein. Plasmid. 49**,** 281-5.

Nieto, C., et al., 2000. Construction of a tightly regulated plasmid vector for *Streptococcus pneumoniae*: controlled expression of the green fluorescent protein. Plasmid. 43**,** 205-13.

O'Sullivan, D. J., Klaenhammer, T. R., 1993. High- and low-copy-number Lactococcus shuttle cloning vectors with features for clone screening. Gene. 137**,** 227-31.

Perez-Arellano, I., et al., 2001. Construction of compatible wide-host-range shuttle vectors for lactic acid bacteria and *Escherichia coli*. Plasmid. 46**,** 106-16.

Ruiz-Cruz, S., et al., 2010. Novel plasmid-based genetic tools for the study of promoters and terminators in *Streptococcus pneumoniae* and *Enterococcus faecalis*. J Microbiol Methods. 83**,** 156-63.
Ruiz-Maso, J. A., et al., 2012. Construction of a plasmid vector based on the pMV158 replicon for cloning and inducible gene expression in *Streptococcus pneumoniae*. Plasmid. 67**,** 53-9.
Simon, D., Chopin, A., 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie. 70**,** 559-66.
Stassi, D. L., et al., 1981. Cloning of chromosomal genes in *Streptococcus pneumoniae*. Proc Natl Acad Sci U S A. 78**,** 7028-32.
Taylor, R. G., et al., 1993. *E. coli* host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. Nucleic Acids Res. 21**,** 1677-8.
Tettelin, H., et al., 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. Science. 293**,** 498-506.
van Alen-Boerrigter, I. J., et al., 1991. Characterization and overexpression of the *Lactococcus lactis* pepN gene and localization of its product, aminopeptidase N. Appl Environ Microbiol. 57**,** 2555-61.

Figure Legends

Fig. 1 - Structural stability of pIL253. Plasmid DNA was extracted from over-night cultures of *S. pneumonia* TIGR4 carrying pIL253, grown in the presence of the selective pressure. Pneumococcal plasmid preparations were compared with the original *L. lactis* plasmid preparation before and after digestion with PstI. M- Molecular weight marker; 1- Original pIL253 preparation; 2- pIL253 extracted from *S. pneumoniae*; 3- Original pIL253 prep digested with PstI; 4- *S. pneumoniae* pIL253 prep digested with Pst1.

Fig. 2 – Compatibility of pIL252 with pLS1. Plasmid DNA was extracted from exponentially growing cultures of *S. pneumonia* TIGR4 carrying pLS1, or pIL252, or both plasmids, in the presence of the respective antibiotics. Digestion of plasmid preparations with PstI is shown. M- Molecular weight marker; 1- pIL252; 2- pLS1; 3- pIL252 + pLS1.

Fig. 3 - Analysis of pIL252 and pIL253 plasmids. Total DNA extracts from TIGR4 strain carrying the indicated plasmids were analyzed. S- SmartLadder linear molecular weight standard (Eurogentec).

Fig. 4 - Expression of pneumococcal RNase R from pIL253. Western blot and RT-PCR analysis of protein and RNA samples extracted at different temperatures (15 ºC and 37 ºC) from *S. pneumoniae* TIGR4 wild type and derivatives as indicated on top of each lane. Details of experimental procedures are described in 'Material and Methods'. (Upper panel) Analysis of *rnr* mRNA levels by RT-PCR. RT–PCR experiments were carried out with primers specific for *rnr* using 100 ng of total RNA extracted from the wild type (WT) and derivatives at 15 ºC or 37 ºC, as indicated at the top of the lanes. The RNase R^- mutant derivative was used as a negative control. RT-PCR with primers specific for 16S rRNA shows that there were no significant variations in the amount of RNA used in each sample. (Lower panel) Analysis of RNase R (~92 kDa) expression by Western immunoblotting. 20 µg of each protein sample were separated in a 7 % tricine-SDS-polyacrylamide gel and blotted to a nitrocellulose membrane. RNase R was detected using specific antibodies. An RNase R^- mutant strain was used as a negative control. A non-specific band (Control) detected with the same antibodies was used as loading control.

Table 1 – List of strains used in this work.

Strain	Relevant markers/Genotype	Source/Reference
E. coli		
DH5α	F' fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17a	{Taylor, 1993 #25}
S. pneumoniae		
708		{Lacks, 1968 #26}
JNR7/87 (TIGR4)		{Tettelin, 2001 #1}
CMA607	TIGR4 carrying pIL253 (Ery^R)	This work
CMA608	TIGR4 carrying pIL252 (Ery^R)	This work
CMA609	TIGR4 carrying pIL253 and pLS1	This work
CMA610	TIGR4 carrying pIL252 and pLS1	This work
CMA611	TIGR4 *rnr*$^-$ (Δ*rnr*-Cm^R)	This work
CMA604	TIGR4 *rnr*$^-$ (Δ*rnr*-Cm^R) carrying pIL253 (Ery^R) expressing RNase R	{Moreira, in press #29}

Figure 1

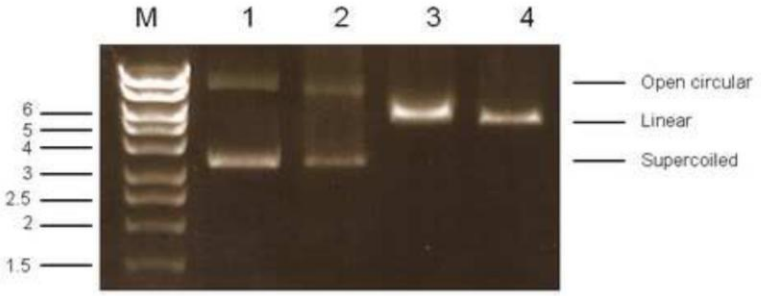
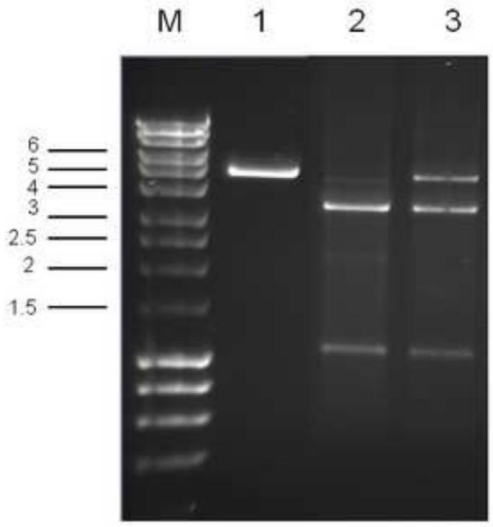


Figure 2



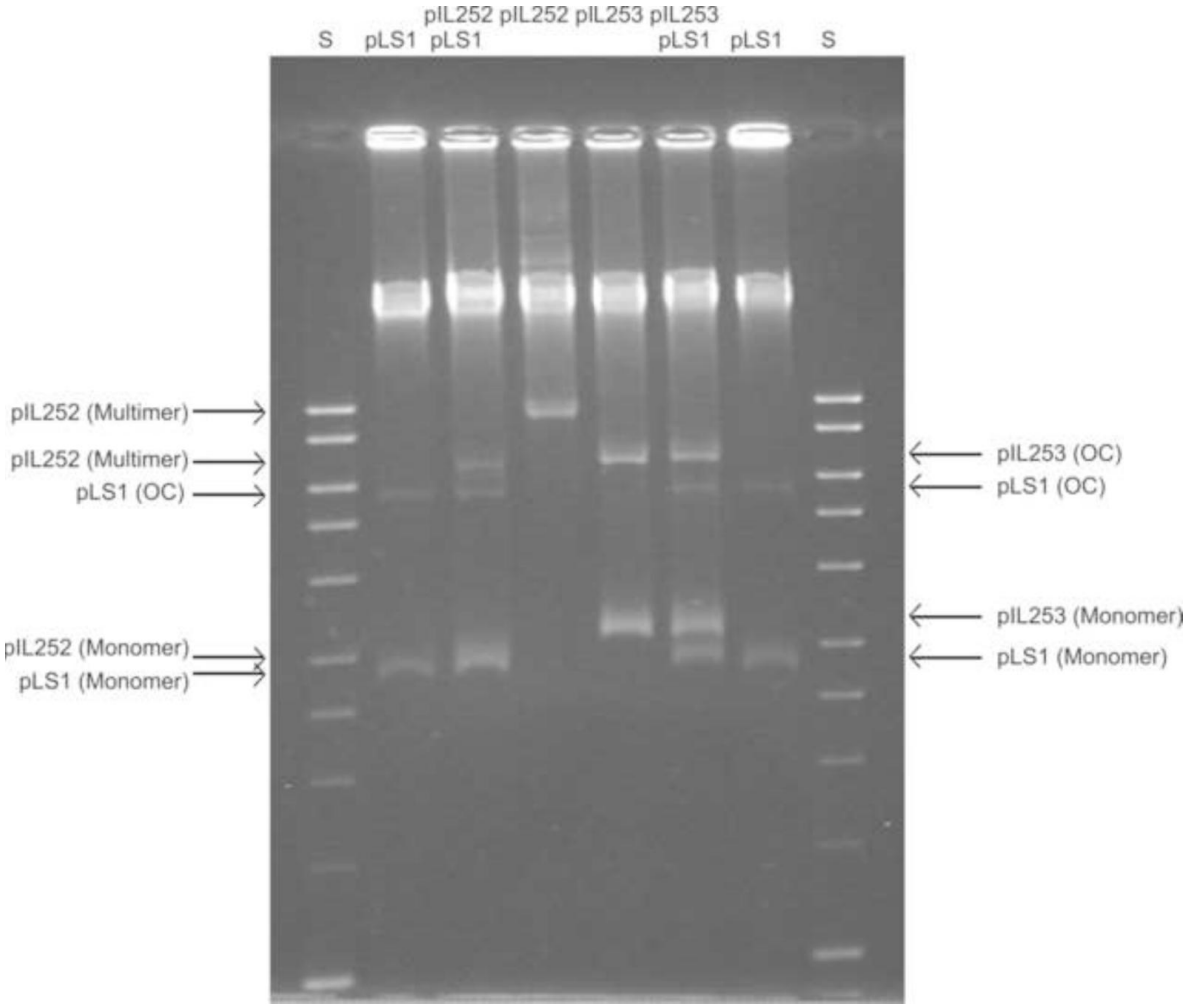
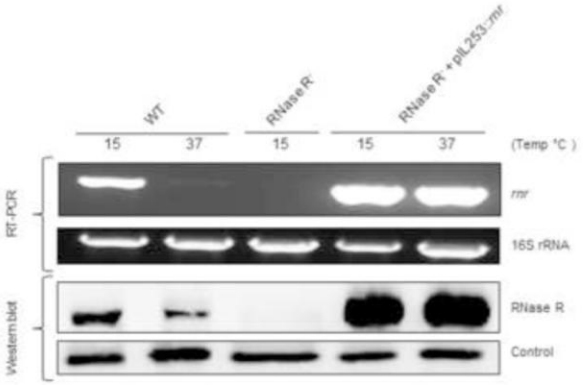

pIL252 pIL252 pIL253 pIL253
S pLS1 pLS1 pLS1 pLS1 S
pIL252 (Multimer)
pIL252 (Multimer)
pLS1 (OC)
pIL252 (Monomer)
pLS1 (Monomer)
pIL253 (OC)
pLS1 (OC)
pIL253 (Monomer)
pLS1 (Monomer)

Figure 4


WT
RNase R
RNase R +pIL253::rnr
15
37
15
15
37
(Temp °C)
RT-PCR
rnr
16S rRNA
Western blot
RNase R
Control