Molecular characterization and exploitation of the temperate Lactococcus lactis bacteriophage r1t
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Chapter 6

The H-N-H endonuclease I-LabI, specified by the bacteriophage r1t group I intron scrI, mediates homing without the need for additional phage-encoded proteins.

Submitted for publication
SUMMARY

The genome of the *Lactococcus lactis* temperate bacteriophage r1t was shown to encompass a functional group I intron, designated scrI. Excision of the 928-nucleotide (nt) intervening sequence results in an in-frame fusion of the messages of the open reading frames (ORFs) 40 and 42. ORF41, contained within scrI, specifies an H-N-H motif-containing endonuclease, I-LabI, that generates a nick in the sense strand of the upstream exon. The enzyme was shown to endow the intron with mobile properties. This homing reaction is independent of additional r1t-encoded products, as was demonstrated by transfer of a plasmid-borne intron to a recipient allele on a second plasmid in the absence of the phage.

INTRODUCTION

Group I introns constitute a phylogenetically widespread class of self-splicing intervening sequences that are characterized by their structural and functional uniformity (Burke, 1988; Lambowitz and Belfort, 1993). Despite a lack of primary sequence homology, they share conserved base-paired regions (P1 through P10) which are involved in the formation of the catalytically active core structure (Cech, 1988; Michel and Westhof, 1990). These so-called ribozymes excise themselves from precursor (pre-) mRNA via a series of transesterification reactions initiated by an exogenous guanosine cofactor, which results in the ligation of the upstream and downstream exon sequences and the generation of a free intron (Cech, 1990).

In many cases, group I introns carry an ORF (Lambowitz and Belfort, 1993). Although its coding region can overlap the intron core structure elements, most ORFs are present as extensions of peripheral loops. The ORFs either specify maturases, facilitating the splicing reaction, site-specific DNA endonucleases, or bifunctional proteins having both activities (Lambowitz and Belfort, 1993). The intron-encoded (I-) endonucleases can be differentiated into four major structural families on the basis of specific amino acid motifs (variations of LAGLI-DADG, GIY-YIG, H-N-H and the His-Cys box). Although most I-endonucleases
generate a double-strand break, some H-N-H endonucleases introduce a nick in one of the strands (Goodrich-Blair and Shub, 1996). The H-N-H motif has also been shown to be present in several group II intron-encoded reverse transcriptase-like proteins, forming part of the Zn$^{2+}$-finger-like domain (Mohr et al., 1993; Gorbalenya, 1994; Shub and Goodrich-Blair, 1994).

It is now well documented that the formation of the double-strand break (DSB) by the I-endonucleases can endow the intervening sequence with mobile properties (Dujon, 1989; Lambowitz and Belfort, 1993). In this process of intron homing, the site-specific cleavage of a cognate intronless-allele near the so-called intron insertion site (IIS) initiates the copying of the intron-sequence. Several mechanisms are thought to account for the post-cleavage steps that are directed towards copying of the intervening sequence into the intronless recipient allele. Most group II introns require the action of a reverse transcriptase (-like) protein to mediate reverse transcription using the (pre-) mRNA as a template (Saldanha et al., 1993). Site-specific intron insertions that are independent of reverse transcription (mainly group I introns) are thought to involve the invasion of DNA ends, generated by the I-endonuclease, in homologous exon sequences of an intron-containing allele to prime DNA repair synthesis. On the basis of the functional requirements and the nature of the obtained recombination products of the various distinct systems, several pathways have been postulated to account for the repair of the DSBs.

Whereas homing by reverse transcription only requires a short sequence homologous to the 5' exon, the DNA repair-mediated mechanism is highly dependent on extensive homology of flanking exon sequences as it requires homologous recombination with the intron-containing template. Thus far, most intron mobilization systems that require the action of an I-endonuclease, need both accessory host and bacteriophage functions (Clyman and Belfort, 1992; Mueller et al., 1996; George and Kreuzer, 1996).

The endonucleolytic characteristics of some of the H-N-H endonucleases notably distinguish them from the other I-endonucleases. To date, it has not been addressed whether the enzymes that generate a single-strand nick also function as homing endonucleases. Here we report, for the first time, that a member of this type of H-N-H endonucleases, I-LabI, specified by the *L. lactis* bacteriophage r1t group I intron scrI, can indeed initiate group I intron inheritance. The r1t mobilization system does not require additional phage-encoded proteins.
RESULTS

Bacteriophage r1t harbours a functional group I intron

Recently, the genome of the *L. lactis* temperate bacteriophage r1t has been sequenced and its fifty putative protein-coding regions have been screened for similarities with sequences present in the databases (this thesis, chapter 2). ORF41 resembles several entries of phage sequences shown to be contained within group I introns. In addition, it is flanked by four sequence elements that are conserved in this type of intervening sequences (P, Q, R, and S; see Fig. 1). These base-paired elements constitute the intron core and are of major importance for the proper folding of the intron and required for its accurate and efficient splicing (Cech, 1988).

Reverse transcription-PCR (RT-PCR) was performed, with oligonucleotides that anneal to the presumed exon sequences (see Fig. 1 and the Experimental procedures), in order to determine whether the primary transcript encompassing ORF41 indeed harbours an excisable intervening sequence. RNA of the lysogenic strain *L. lactis* R1 was isolated 1.5 hours after UV-induction of r1t and used for the synthesis of cDNA of the region of interest (primer I1). The cDNA thus obtained was subsequently amplified by PCR (primers I1 and I2). As a control, r1t genomic DNA was PCR-amplified using the same primers. The amplified cDNA product was approximately 900 bp smaller than that obtained when genomic r1t DNA was used as a template, indicating that the sequence encompassing ORF41 is indeed spliced out of the mRNA in vivo (results not shown). In addition, it can be concluded that the splicing reaction resulted in the joining of the transcripts derived from the flanking exon regions to form one contiguous messenger.

To determine the 5’- and 3’ splice sites and, thereby, the size of the intervening sequence, the RT-PCR amplification product was subcloned (pHI01) and subjected to nucleotide sequencing. Comparison of its sequence with that of the r1t genome revealed that 928 nt had been removed from the primary transcript (Fig. 1). As is the case for most group I introns, a U and G residue precede the 5’- and 3’ splice sites, respectively. Upon splicing, the ORF40 and 42 coding regions are fused in-frame (between corresponding r1t residues 23379 and 24308). As their combined deduced amino acid sequence exhibits significant similarity with the minor tail protein of mycobacteriophage L5 (Hatfull *et al.*, 1993; accession Number Z18946), this fused ORF probably encodes a structural component of the phage.
Figure 1. Secondary structure model of the r1t group intron scr1. The base-paired regions (P1, P3-P10), conserved P,Q,R and S sequences and ORF41, designated ILabl (boxed) are indicated. Flanking exon sequences are indicated by lower-case letters, arrows depict the 5' and 3' splice-sites. The scr1 residues that were mutated in pH15, are encircled.
Comparative sequence analysis was used to make a model of the secondary structure of the r1t intron, which we designate scrI (Fig. 1). On the basis of two additional hairpins (P7.1 and P7.2) located between the stems P7 and P3, scrI is categorized in subgroup IA2 (Michel and Westhof, 1990). ORF41 constitutes an extension of the P6a peripheral stem and loop. The conserved base-paired elements R and S have been shown to be critical for splicing via the group I mechanism (Burke, 1988). To confirm the existence of a similar pairing in the r1t structure and its importance for splicing, pHI15 was constructed (see Fig. 2 for a schematic representation of the plasmids used in this study). This plasmid harbours a copy of scrI, in which two point mutations are introduced in element R (see Fig. 1 for exact positions), and flanking exon regions downstream of the inducible P<sub>nisA</sub> promoter.

**Figure 2. Schematic representation of the r1t sequences present on the plasmids used in this study. Intron scrI is indicated by a grey box, the up- (A and A<sub>1</sub>) and downstream (B and B<sub>1</sub>) exon sequences are represented by open boxes. The I-LabI gene is depicted by an arrow. The scrI residues that were mutated in pHI05 (*) and the deletion within the I-LabI gene in pHI05 (▼) are indicated. P: inducible P<sub>nisA</sub> promoter.**
The plasmid was introduced in *L. lactis* NZ3900 to enable nisin-induced transcription of the region. RNA was isolated from this strain 2.5 hours after induction. As the size of the PCR-amplified cDNA (using the primers described above) was identical to that of the PCR product obtained with pHI15 DNA as template, it was concluded that the mutations blocked the splicing reaction (Fig. 3; lanes 3 and 7). The control plasmid pHI04, carrying the same DNA fragment without the two mutations, gave normal splicing (Fig. 3; lane 1).

**Figure 3.** Splicing of the rII group I intron scrI. The products obtained by PCR amplification of reverse transcribed mRNA isolated from *L. lactis* NZ3900 (pHI04), *L. lactis* NZ3900 (pHI05) and *L. lactis* NZ3900 (pHI15) are shown in lanes 1, 2, and 3, respectively. Lanes 4, 5, 6, and 7 contain PCR fragments obtained when pHI01, pHI04, pHI05 and pHI15 DNA was used as a template, respectively. The sizes of the products are indicated in the right margin.

**The product of ORF41 is not required for splicing in vivo**

To examine whether the product of ORF41 is required for excision of scrI from the primary transcript, we tested for splicing in strains *L. lactis* NZ3900 (pHI04) and *L. lactis* NZ3900 (pHI05). Whereas plasmid pHI04 contains the complete intron and flanking exon regions downstream of *P*<sub>nisA</sub>, its derivative pHI05 carries a 377-bp deletion in ORF41, thereby introducing a stop codon after codon 10. It appeared that the two fragments obtained by RT-PCR were of similar size (Fig. 3; lanes 1 and 2), and smaller than those obtained when pHI04
and pHI05 DNAs were PCR-amplified (Fig. 3; lanes 5 and 6). Their sizes were identical to the amplification product of intronless splice site-containing pHI01 DNA (Fig. 3; lane 4). These results indicate that splicing had also occurred in the strain in which the ORF41 product had been inactivated by truncation.

**ORF41 specifies an H-N-H endonuclease, I-LabI**

The deduced amino acid sequence of ORF41 is similar to the deduced sequences of several intron-encoded (putative) endonucleases of other bacteriophages (Fig. 4).

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**Figure 4.** Alignment of the deduced amino acid sequence of r1t ORF41 and various phage intron ORFs. Amino acids are boxed if four or more of the depicted proteins contain identical residues at corresponding positions. Sequences are from the following phages: LLH (accession Number L37351), SP82 (I-HmuII; accession Number U04812), SPP1 (ORF36.1; accession Number X67865), phi-E (accession Number U04813) and SPO1 (I-HmuI; accession Number P34081).
It shows a high degree of similarity with the derived products of group I intron ORFs of the virulent *Lactobacillus delbrueckii* subsp. *lactis* bacteriophage LL-H (Mikkonen et al., 1995) and phages phi-E, SPP1, SP01 and SP82 of *Bacillus subtilis*. On the basis of the conserved H-N-H (-like) domains, these proteins can be grouped in a family of (putative) endonucleases (Shub and Goodrich-Blair, 1994).

As two members of this family, I-*Hmu*I and I-*Hmu*II, have been shown to introduce a nick near the splice junction, we examined whether the product of ORF41, designated hereafter as I-*.Lab*I (Intron-encoded endonuclease of *lactococcal* bacteriophage r1t), could cleave pHIO1. The I-*.Lab*I gene was placed under the control of P*nisA* on plasmid pHIO2 and introduced in *L. lactis* NZ3900 (pHI01). Both plasmids can be discriminated by restriction with *Hind*III which results in fragments of 3868 and 1437 bp (pHI01) and of 5565 bp (pHI02). Two hours after induction of I-*.Lab*I expression, covalently closed circular (ccc) plasmid DNA was isolated. As can be seen from figure 5, expression of I-*.Lab*I resulted in a rapid decrease in the amount of pHIO1 DNA as compared to the situation in which I-*.Lab*I expression was not induced. After twenty-one hours after induction, hardly any pHIO1 could be recovered. The observed minor decrease of pHIO1 DNA in the uninduced culture can be ascribed to a low level of leakage of P*nisA* (data not shown). We interprete these results to indicate that I-*.Lab*I cuts the pHIO1 molecules after which they are further degraded by (cytoplasmic) (exo-) nucleolytic activity.

Figure 5. *Effect of I-*.Lab*I expression on intronless splice site-containing pHIO1*. An overnight culture of *L. lactis* NZ3900 (pHI01, pHI02) was diluted 100-fold and samples were grown for the indicated amount of time with (+) or without (-) nisin added at an OD of the cultures of 0.5. Plasmid DNAs were isolated and digested with *Hind*III prior to loading on an 0.8% agarose gel in order to distinguish between linearized pHI02 (5565 bp) and pHIO1 (two fragments: 3868 and 1437 bp). M, molecular size marker: *B. subtilis* SPP1 phage DNA digested with *EcoRI*.  

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I-LabI introduces a nick in the sense strand near the splice site junction

To determine the endonucleolytic properties of I-LabI, total DNA was isolated from L. lactis NZ3900 (pHI01, pHI02) at various time points after induction of I-LabI. Southern hybridization analysis showed that induction of I-LabI expression resulted in the accumulation of open (nicked) circular (oc) pHI01 DNA (Fig. 6A).

The site at which the nick is generated was determined by primer extension of oc pHI01 DNA of induced L. lactis NZ3900 (pHI01, pHI02) isolated from an agarose gel. Two strand-specific primers, annealing to both flanking exon sequences were used for two independent extension reactions. It appeared that only the sense strand-specific primer gave rise to an extended
product. Running it parallel to a nucleotide sequence ladder obtained with the same primer enabled the precise mapping of the nick site (Fig. 6B). The single-strand break is introduced three bp upstream of the intron insertion site (see below), between two C residues (residues 23376 and 23377 of the published r1t nucleotide sequence).

**I-LabI mediates homing without the need for additional r1t-encoded proteins**

To determine whether I-LabI endows the intron with mobile properties, we tested for homing between a plasmid-borne intron donor (pHI04) and an intronless recipient allele on a second plasmid (pHI11). To discriminate between homing and recombination between homologous regions, we used pHI05 as a control. Plasmid DNA was isolated from *L. lactis* NZ3900 (pHI11, pHI04) and *L. lactis* NZ3900 (pHI11, pHI05). Southern hybridization using *Hind*III digestion and an exon-specific probe was used to discriminate between pHI11, pHI04 and pHI05 (Fig. 7, lanes 1-3; 2007 bp, 4925 bp and 4552 bp, respectively). The hybridization pattern in Fig. 7 shows that expression of I-LabI in *L. lactis* NZ3900 (pHI11, pHI04) results in the enlargement of the hybridizing pHI11-specific *Hind*III fragment to the expected size (Fig. 7, lane 4; 2007 + 928 = 2935 bp). Nucleotide sequencing of the enlarged pHI11 insert revealed that the intron had, indeed, been inserted in the splice junction, designated hereafter as intron insertion site (IIS). As intron inheritance was not observed in several independent attempts in *L. lactis* NZ3900 (pHI11, pHI05) (Fig. 7, lane 5 shows one example), we conclude that the observed mobilization of scrI is due to the action of I-LabI.
**Homing is dependent on the length of homologous flanking exon regions**

In order to determine whether the efficiency of homing is dependent on the length of the flanking homologous exon regions on the donor and recipient plasmids, single colonies of *L. lactis* NZ3900 (pHI04, pHI01) and *L. lactis* NZ3900 (pHI04, pHI11) were screened for homing. As can be seen in Fig. 2, plasmids pHI01 and pHI11 only differ in the lengths of the bordering exon sequences; pHI11 contains 700 additional bp as compared to pHI01. The efficiency of homing strongly depends on the length of the sequences flanking the IIS (Table 1). The percentage of pHI01 plasmids that inherited scrI was about 15. When pHI11 was used as a recipient, however, homing was observed in more than 70% of the colonies tested.

In order to determine whether a splicing-deficient intron can also be homed, we used strains *L. lactis* NZ3900 (pHI15, pHI01) and *L. lactis* NZ3900 (pHI15, pHI11). It was shown that the splicing incompetent scrI is also mobilized. Also in this case, the presence of a larger exon region positively influenced homing efficiency. Interestingly, when pHI15 was used as an intron-donor, the percentage of homing was even higher than that observed with the wild-type intron donor pHI04 (Table 1). Homing from pHI15 into pHI01 and pHI11 was about 20% higher than from pHI04.

**Table 1. Plasmid-to-plasmid homing efficiencies.**

<table>
<thead>
<tr>
<th>Donor plasmid</th>
<th>Recipient plasmid</th>
<th>Homing (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHI05</td>
<td>pHI11</td>
<td>0%</td>
</tr>
<tr>
<td>pHI04</td>
<td>pHI11</td>
<td>66%</td>
</tr>
<tr>
<td>pHI04</td>
<td>pHI01</td>
<td>7%</td>
</tr>
<tr>
<td>pHI15</td>
<td>pHI11</td>
<td>47%</td>
</tr>
<tr>
<td>pHI15</td>
<td>pHI01</td>
<td>8%</td>
</tr>
</tbody>
</table>
DISCUSSION

In this study, the genome of the lactococcal temperate bacteriophage r1t was shown to contain a functional group I intron, scrI. The intron appears to be located in a gene determining some unknown structural component of r1t. Upon splicing, the 928 nt comprising intervening sequence is removed from the primary transcript. As a result, the ORF40 and 42 messages are joined to form one contiguous transcript. This explains the apparent lack of signals required for translation of ORF42 (see chapter 2). The predicted structural element R was shown to be essential for splicing, as introduction in R of two point mutations completely abolished excision of scrI from the primary transcript. The product of the I- LabI gene present on scrI, was shown not to be involved in the splicing process.

On the basis of amino acid sequence homologies, I- LabI can be regarded as a member of the H-N-H endonuclease family. The enzyme was shown to have endonucleolytic properties similar to those of other members of this family. I- LabI generates a nick in vivo in the sense strand of a plasmid containing an intronless splice junction. The nick occurred three bp upstream of the splice junction. Under the experimental conditions used, the antisense strand was unaffected. The H-N-H endonucleases I- HmuI and I- HmuII have also been shown to introduce a single-strand nick in the template strand near the splice junction. However, the sites at which the targets are nicked clearly differ. Whereas the SP01 intron-encoded endonuclease (I- HmuI) cleaves its target 4 bp downstream of the splice junction, the SP82 I- HmuII nick site is situated 52 bp downstream of the splice junction.

Like the other I-endonucleases, the H-N-H endonucleases that generate a single-strand nick have been hypothesized to endow the intron with mobile properties. So far, however, homing has never been demonstrated to be initiated by this type of H-N-H nucleases. This study provides the first evidence for the involvement of such an H-N-H endonuclease in intron mobility. By using the plasmid-to-plasmid homing assay we were able to show that scrI can be copied into an intronless allele, provided that an intact copy of the I- LabI gene was present. Moreover, it appeared that no additional phage-encoded proteins are required for mobilization.

The observed effect of the length of the homologous flanking regions on the homing efficiency indicates that homing of scrI most likely proceeds via a DNA repair-mediated mechanism rather than by reverse transcription. The absence of conserved domains normally present in reverse transcriptases in I- LabI would be in agreement with this assumption.
Although the precise mechanism that underlies the copying of scrI awaits elucidation, we show that I-LabI is the only r1t-encoded protein required for scrI mobility. The copying of a plasmid-borne intron into its cognate intronless recipient allele on a second plasmid implicates that, if the system would require additional factors (e.g. a resolvase in the DSBR pathway), these should be host-encoded.

Intron homing mediated by members of the other families of I-endonucleases have been shown to involve the double-strand cleavage of the intronless-allele near the intron insertion site. The double-strand break is thought to initiate DSB repair resulting in the copying of the intron sequence. It is as yet unclear whether the single-strand nick generated by some H-N-H endonucleases suffices for homing, or that the opposite strand also undergoes cleavage when the host would be infected with the complete bacteriophage chromosome. Cleavage of the antisense strand could be dependent on the presence of the mRNA derived from the region encompassing the intron. An RNA cofactor has been shown to be required for cleavage of both strands of target DNA by the H-N-H motif-containing reverse transcriptase-like protein encoded by the group II intron al2 of yeast mtDNA (Zimmerly et al., 1995). The sense strand is cleaved first by al2 protein-stabilized al2 RNA, after which the al2 protein cleaves the antisense strand. If the generation of a double-strand break is also a prerequisite for scrI homing, the catalytic activity of the excised intron RNA is probably not required, as homing was observed with a splicing-incompetent variant of scrI. Rather, the unspliced pre-mRNA could have a role in the mobilization process. Whereas in the wild-type situation the scrI-containing mRNA will rapidly undergo splicing, transcription of the region encompassing splicing-deficient scrI results in the generation of RNA transcripts that remain unspliced. In this scheme, the observed increase in the homing efficiency with the splicing-deficient intron donor could be due to a longer half life of the unspliced transcript.

The presence of the H-N-H motif in several intron-encoded reverse transcriptases and endonucleases has led to the assumption that these proteins could be evolutionary related. The data presented here provide further evidence that, despite the presumed differences in the mechanisms underlying homing, the proteins are also functionally related. Although only few representatives have been studied, the enzymes appear to generate a nick near the IIS. Whether the H-N-H endonucleases also form a ribonucleoprotein directed towards a target in the opposite strand, as has been shown for the al2 protein, and what determines the preference for the strand that undergoes nicking by the protein component, awaits further elucidation.

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EXPERIMENTAL PROCEDURES

Bacterial strains, media and transformation

The bacterial strains used in this study are listed in Table 2. *E. coli* was grown in TY broth (Rottlander and Trautner, 1970) or on TY (1.5%) agar plates. *L. lactis* was grown in glucose 1/2M17 broth (Terzaghi and Sandine, 1975), or on glucose 1/2M17 (1.5%) agar plates. Erythromycin and chloramphenicol were used at 5 µg/ml (*L. lactis*), ampicillin was added at a final concentration of 40 µg/ml (*E. coli*). *E. coli* was transformed using the method of Mandel and Higa (1970). Plasmids were introduced in *L. lactis* by electrotransformation (Leenhouts and Venema, 1993).

DNA techniques

Plasmid DNA was isolated by the method of Birnboim and Doly (1979), total cellular DNA as described by Leenhouts et al. (1991). Restriction enzymes and T4 DNA ligase were obtained from Boehringer (Mannheim, Germany) and used according to their instructions. For nucleotide sequence analysis the dideoxynucleotide chain termination method (Sanger et al., 1977) was used with §-dATP (Amersham International Inc., Amersham, United Kingdom) and the T7 sequencing kit and protocol (Pharmacia, Uppsala, Sweden).

RT-PCR

RNA was isolated from *L. lactis* as described by van Asseldonk et al. (1993). Synthesis of cDNA was performed using the reverse transcription (AMV) Kit and protocol of Boehringer. mRNA (2 µg) was reverse transcribed using 50 ng of synthetic oligonucleotide. The cDNA thus obtained was subsequently amplified by PCR using the GeneAmp2400 system (Perkin Elmer Corp., Norwalk, Co., USA) in 35 of the following cycles: 94 °C for 1 min; 50 °C for 1 min; 73 °C for 2 min.
Table 2. Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant features</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strain</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>L. lactis</em> NZ3900</td>
<td>NZ3000 derivative; pepN::nisRK</td>
<td>de Ruyter <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><em>L. lactis</em> NZ9700</td>
<td>Nisin-producing transconjugant containing Tn5276</td>
<td>Kuipers <em>et al.</em> (1993)</td>
</tr>
<tr>
<td><em>L. lactis</em> R1</td>
<td>r1t lysogen</td>
<td>Lowrie (1974)</td>
</tr>
<tr>
<td><em>E. coli</em> MC1000</td>
<td>araD139 lacX74 Ω(ara leu)7697 galU galK strA</td>
<td>Casadaban and Cohen (1980)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pNZ8010</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, carrying the nisin-inducible promoter P&lt;sub&gt;nisA&lt;/sub&gt;</td>
<td>de Ruyter <em>et al.</em> (1996)</td>
</tr>
<tr>
<td>pIL253</td>
<td>Em&lt;sup&gt;R&lt;/sup&gt;, pAMβ derivative</td>
<td>Simon and Chopin (1988)</td>
</tr>
<tr>
<td>pH101</td>
<td>Em&lt;sup&gt;R&lt;/sup&gt;, pIL253 derivative carrying a 287-bp splice junction-containing r1t fragment</td>
<td>This study</td>
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<tr>
<td>pH111</td>
<td>Em&lt;sup&gt;R&lt;/sup&gt;, pIL253 derivative carrying a 987-bp splice junction-containing r1t fragment</td>
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<td>pH102</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, pNZ8010 derivative carrying the I-Lab1 gene</td>
<td>This study</td>
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<td>pH104</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, pNZ8010 derivative carrying a 1915-bp srl-containing fragment of r1t</td>
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<td>pH105</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, pH104 derivative in which 377 bp of the I-Lab1 gene are deleted</td>
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<td>pH115</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;, pH104 derivative with two point mutations within srl</td>
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*Em<sup>R</sup>, Cm<sup>R</sup> resistances to erythromycin and chloramphenicol, respectively*

**Oligonucleotides**

Synthetic oligonucleotides were synthesized using an Applied Biosystems 392A DNA synthesizer (Applied Biosystems Inc., Foster City, Calif.). The corresponding positions in the r1t nucleotide sequence (EMBL/GenBank/DDBJ Nucleotide Sequence Data Library Accession Number U38906) of the upper-case sequences are given in brackets. The upper-case nucleotides printed in italic represent introduced mutations. Underlined lower-case residues
indicate relevant restriction enzyme sites. The oligonucleotides had the following 5'-3' nucleotide sequences: I₁: gcg cgg aTC CTG CAA ATG CGT ATA TGG AAA CTG (positions 23161 to 23185), I₂: gcg ctc tag aAA ATG TTG TTG ATG CTT TCT TCG C (positions 24475-24452), I₃: gcg cgc gta cgc gga tcc cgg gga ATG CCA AAG GAA TAT CAG G (positions 22887 to 22904), I₄: gcg cgc gtc gac tct agA CTG GTT TTA AGC CTG AGA C (positions 24801 to 24784), I₅: gcg cgg atc caa gga gga CTG ATT ATG TGG GTA AAA ATT G (positions 23543 to 23565), I₆: gcg cct gca gTG TTT CGA TAG TCG TTA CAC (positions 24132 to 24113), I₇: tta tag acT AGT TAT TAT TTC TTG C (positions 23581 to 23565), I₈: aaa att tct AGA AAT AAT AAC Tag TAA ATA AAT GAA AAC G (positions 23568 to 23581 and 23587 to 23598), I₉: CTG TTT CGA TAG TAC TAG TTA CAC TTT TAT G (positions 24133 to 24106), I₁₀: AGT GTA acT AGT ATC ACA ACA G (positions 24112 to 24133).

Plasmid constructions

Plasmids pHI01 and pHI11 were constructed by subcloning BamHI/XbaI restricted splice site-containing fragments obtained by RT-PCR in the corresponding restriction enzyme sites of pIL253. The primers used for reverse transcription of the r1t RNA and the subsequent PCR were: I₁ and I₁/I₂, and I₄ and I₅/I₆, respectively. For the construction of pHI02 and pHI04, r1t DNA was PCR amplified using primers I₅/I₆ and I₃/I₄, respectively. After digestion with BamHI/PstI and BamHI/XbaI the fragments were subcloned in the corresponding sites of pNZ8010. Plasmids pHI05 and pHI15 were obtained by performing a two-step PCR strategy. In plasmid pHI05, the two fragments obtained with primers I₅/I₆ and I₃/I₄ were digested with BamHI/Spel and SpeI/XbaI, respectively. Both fragments were fused upon their subcloning in BamHI/XbaI-digested pNZ8010. Using the same procedure with the two complementary mutation primers I₉ and I₁₀, also harbouring an unique SpeI restriction enzyme sites, in combination with either primer I₅ or I₄, respectively, resulted in plasmid pHI15. The nucleotide sequences of the PCR fragments present in the respective plasmids were verified by nucleotide sequencing. For a schematic representation of the plasmids, see Fig. 2)

Nisin induction

Transcription of the region downstream of Pₙₙₐ in pNZ8010 derivatives in L. lactis NZ3900 was induced by the addition to exponentially growing cells (OD₆₀₀ of 0.3) of 1/1000 of the supernatant of an overnight culture of the nisin-producing L. lactis NZ9700.
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Mapping of I-LabI cleavage sites

Total plasmid DNA was isolated from the cultures one hour after nisin induction and run on an 0.8% agarose gel. The oc splice junction-containing plasmid pH11 DNA band was isolated and purified by phenol/chloroform extraction. Purified DNA served as template for two separate strand-specific extensions using the CircumVent Thermal Cycle Dideoxy DNA Sequencing Kit (New England Biolabs Inc.). The reaction conditions were essentially as described by the supplier but the dideoxynucleotide triphosphates were replaced by a d(CGT)TP mix (0.25 mM) and 2 µM [35S]-dATP). The extension products were separated by electrophoresis on a 6% polyacrylamide urea gel next to a DNA sequence ladder of the region, obtained with the same primer. The gels were dried and analyzed by autoradiography.

Southern transfer and DNA hybridization

After electrophoresis on a 0.8% agarose gel in TBE buffer (Sambrook et al., 1989), DNA fragments were transferred to a Qiabrane nylon plus filter (Diagen, GmbH, Düsseldorf, Germany) by the method of Southern, as modified by Chomczynski and Quasba (1984). The enhanced chemiluminescence (ECL) labelling and detection system and accompanying protocols (Amersham International, Amersham, UK) were used for labelling of the probes and for hybridization.

Plasmid-to-plasmid homing assay

L. lactis NZ3900 was transformed with recipient and donor plasmids, and plated to single colonies. Plasmid DNA was isolated and restricted with HindIII. Donor, recipient and recipient plasmids that had inherited the intron were distinguished on the basis of the sizes of the HindIII fragments.

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REFERENCES

References are listed in chapter 9.