Inducible gene expression mediated by a repressor–operator system isolated from Lactococcus lactis bacteriophage r1t

Arjen Nauta, Douwe van Sinderen,† Harma Karsens, Egbert Smit, Gerard Venema* and Jan Kok
Department of Genetics, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.

Summary

A regulatory region of the temperate Lactococcus lactis bacteriophage r1t chromosome has been cloned and characterized. It encompasses the two divergently oriented genes rro, encoding the phage repressor, and tec. Both genes, of which the transcription start sites have been mapped, are preceded by consensus −35 and −10 promoter sequences. The region contains three 21 bp direct repeats with internal dyad symmetry which probably act as operators. Two of these repeats partially overlap the two promoter sequences. The distant third repeat is located within the tec coding sequence. Gel mobility shift assays demonstrated that Rro specifically binds to this sequence. To study possible transcriptional regulation of the region, a lacZ translational fusion with an open reading frame following tec was constructed. Under conditions that favour the lysogenic life cycle of r1t, β-galactosidase activity was very low. Expression of the lacZfusion could be induced 70-fold by the addition of mitomycin C at a concentration which promotes the switch of r1t from the lysogenic to the lytic life cycle. In non-induced cells, promoter activity was repressed by Rro, as a frameshift mutation in rro resulted in constitutive expression of the lacZgene fusion.

Introduction

Gene-expression systems in which the expression of a certain gene can be controlled can bypass the possible lethality of (heterologous) gene products. For the Gram-positive lactic acid bacterium Lactococcus lactis two inducible systems have been described so far. The lac promoter upstream of the lactococcal lac genes is regulated by the LacR repressor and can be used for lactose-inducible gene expression (van Rooijen et al., 1992). Replacing glucose by lactose in the growth medium results in a sixfold induction of transcription of genes downstream of the lac promoter in cells containing an expression vector based on this system. In a further refinement of the system, it has recently been combined with a gene expression system based on the T7 RNA polymerase and its cognate promoter (Wells et al., 1993). For that purpose, the gene encoding T7 RNA polymerase was placed under the control of the lac promoter. Upon growth on lactose of cells containing this system, the T7 RNA polymerase gene is expressed. As a result, expression of genes under the control of the T7 promoter is induced. A disadvantage of the T7/lac system is the fact that it is encoded by three different plasmids, which have to be introduced in strains used in the food industry.

An easily manageable food-grade expression system allowing carefully controlled production of (heterologous) proteins (e.g. antimicrobials and enzymes involved in cheese-flavour development) would be of great interest. We set out to develop such a system on the basis of the regulatory region of a temperate L. lactis bacteriophage. Although many reports on the presence of temperate bacteriophages in lactococci have been published, little is known about the molecular basis for control and maintenance of the lysogenic state in these bacteria. A putative regulator gene, bpi (for BK5-T promoter inhibitor), of the temperate L. lactis subsp. cremoris phage BK5-T has been cloned and sequenced (Lakshmidevi et al., 1990). The bpi gene product inhibited the activity of a number of BK5-T promoters. The mechanism by which the product of bpioperates is unknown. Recently, a putative repressor protein of the L. lactis bacteriophage Tuc2009 has been described (van de Guchte et al., 1994). Although this protein shows significant homology with repressor proteins of other bacteriophages, its ability to control gene expression has not yet been demonstrated.

In this study we report the characterization of a regulatory region of the genome of the temperate small isometric-headed L. lactis subsp. cremoris bacteriophage r1t. The data presented show that a specific DNA fragment
of bacteriophage r1t contains a gene (rro) that specifies a DNA-binding protein capable of repressing gene expression from a promoter on the same fragment. Using lacZ as a reporter gene, it is shown that this regulatory region of bacteriophage r1t can be exploited for inducible gene expression in L. lactis.

Results

The r1t regulatory region

We have recently determined the complete 33350 bp nucleotide sequence of the temperate L. lactis subsp. cremoris bacteriophage r1t (see the accompanying paper by van Sinderen et al., 1996). All of the identified open reading frames (ORFs) had the same orientation, except for a cluster of three consecutive ORFs (ORFs 1-3). ORF3 (designated rro hereafter) has an orientation opposite to that of ORF4 (tec) and ORF5. The non-coding region between the oppositely oriented rro and tec (Fig. 1) contained two divergently oriented sequences, P1 and P2, identical to the consensus promoter sequence used by the vegetative form of the L. lactis RNA polymerase (van de Guchte et al., 1992).

The deduced amino acid sequence of rro shows significant similarity (78.8% identity) with the putative repressor Cl of the L. lactis bacteriophage Tuc2009 (van de Guchte et al., 1994b). Rro, which has a calculated molecular mass of 31.5 kDa, also shows similarity with the C-terminal parts of other phage-encoded repressor proteins such as the Cl repressor of the Escherichia coli bacteriophage 434 (Nikolinkov et al., 1984) and the C2 repressor of E. coli bacteriophage P22 (Sauer et al., 1981). In addition, significant homology was found with various regulator proteins involved in SOS induction. In Fig. 2, the similarities of Rro with the phage repressors and the proteins LexA (Horii et al., 1981) and DinR (Raymond-Denise and Guillen, 1991), which repress a set of damage-inducible genes in E. coli and Bacillus subtilis, respectively, are indicated. These similarities suggested that rro (for repressor of r-one) might specify the bacteriophage r1t repressor protein.

As most bacteriophage-specific repressor proteins contain a so-called helix-turn-helix motif involved in binding of the protein to its DNA target, we compared the deduced amino acid sequence of rro with a ‘master set’ of pre-aligned amino acid sequences known to adopt a helix-turn-helix conformation. By using the amino acid versus position score matrix (weight matrix) developed by Dodd and Egan (1990), which is based on the probability of a specific amino acid being present at a certain position within the motif, a score of 2157 was obtained for a stretch of 20 amino acids (Fig. 3). This score suggests that the sequence is likely to be involved in the binding of a specific DNA target.

The topological equivalent of the lambda cro gene, tec, could specify a protein of 80 amino acids with a calculated molecular mass of 8981 Da. Upstream of tec, a potential ribosome binding site is present that shows strong complementarity to the L. lactis 3'-16S rRNA sequence...
Inducible gene expression in Lactococcus lactis

Fig. 2. Alignment of Rro with the putative CI repressor of L. lactis bacteriophage Tuc2009, the C-terminal parts of the repressors CI and C2 of the E. coli bacteriophages 434 and P22, and the proteins LexA and DinR. Identical (*) and similar (#) amino acids between Rro and CI of Tuc2009 are indicated above the sequences, and amino acids that are identical (*) or similar (#) in all five sequences are indicated beneath the sequences. Conserved residues, between which autodigestion has been shown to take place in LexA (Little, 1993), are shaded. The putative helix-turn-helix of Rro is underlined.

\( \Delta G = -19.4 \text{ kcal mol}^{-1} \) (Tinoco et al., 1973; Chiaruttini and Millet, 1993). Although the method described above does not select a helix-turn-helix motif, the deduced amino acid sequence of tec, like that of cro, contains consensus amino acids at particular positions in helix-turn-helix motifs (Fig. 3).

\( P_1 \) and \( P_2 \) function as transcriptional start signals

The transcription initiation sites of ro and tec were determined by primer extension mapping. RNA was isolated from L. lactis cells harbouring either plR12 or plR13 (see below for a description of both plasmids).

Fig. 3. Alignment of the putative helix-turn-helix motifs in Rro and Tec with those in the putative transcriptional control protein CI of the L. lactis bacteriophage Tuc2009 and the repressors CI and C2 of the E. coli bacteriophages 434 and P22, and the proteins LexA and DinR. Identical (*) and similar (#) amino acids between Rro and CI of Tuc2009 are indicated above the sequences, and amino acids that are identical (*) or similar (#) in all five sequences are indicated beneath the sequences. Conserved residues, between which autodigestion has been shown to take place in LexA (Little, 1993), are shaded. The putative helix-turn-helix of Rro is underlined.
Fig. 4. Determination of the transcriptional start sites from P1 and P2 by primer extension mapping. Lanes PE show the extended products using primers RPE (P1) and TPE (P2). The nucleotide sequence ladders, obtained with the corresponding primers, were run in parallel. The relevant nucleotide sequences are indicated in the right-hand margins. The −10 sequences (shaded) and 5' ends of the P1 and P2 transcripts (asterisks) are indicated.

The nucleotide sequences of the primers (RPE and TPE, respectively) that were used for the independent reverse transcriptions are depicted in the Experimental procedures. The studies confirmed the position of the two postulated promoters P1 and P2. The size of the extension products indicated that the transcription start site from P1 is at the A at position 2757. The T at position 2846 specifies the 5' end of the mRNA produced from P2 (Fig. 4).

Rro binds to specific DNA sequences in the regulatory region

The intergenic region between rro and tec contains two almost perfectly matching 21 bp direct repeats with internal dyad symmetry, O2 and O3. The distance between both their centres constitutes 24 bp (Fig. 1). Because O2 overlaps the −35 sequence of the promoter P2, and O3 overlaps that of P1, these sequences may function as binding sites for the r1t repressor. A third putative operator site, O1, is situated within the coding region of tec at a distance of 402 bp from O2. Alignment of their six half-sites enabled the designation of an 11 bp-long consensus half-site (Fig. 5).

In order to determine whether Rro is able to bind DNA and specifically recognize one of the depicted 21 bp sequences, gel mobility shift assays were performed. The rro gene was amplified by the polymerase chain reaction (PCR) and placed under the control of the IPTG-inducible operator.

<table>
<thead>
<tr>
<th>Operator</th>
<th>Half-site Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1</td>
<td>A A C T A G C C A A T</td>
</tr>
<tr>
<td></td>
<td>A A C T T G A C A A A</td>
</tr>
<tr>
<td>O2</td>
<td>A A C T A T C C A A T</td>
</tr>
<tr>
<td></td>
<td>A A C T T G A C A A A</td>
</tr>
<tr>
<td>O3</td>
<td>A A C T T T C C A A A</td>
</tr>
<tr>
<td></td>
<td>A A C T T G T C A A T</td>
</tr>
<tr>
<td>Consensus</td>
<td>A A C T T G T C A A T</td>
</tr>
</tbody>
</table>

© 1996 Blackwell Science Ltd, Molecular Microbiology, 19, 1331–1341
Fig. 6. Gel mobility-shift assays performed with the synthetic double stranded 21 bp DNA fragment 0₁ (lanes 1–3), and a 21 bp negative-control fragment C (lanes 4–6). The [γ-32P]-ATP-labelled DNA fragments were incubated for 15 min at room temperature with E. coli cell-free extracts either containing Rro (E. coli (pAG58SR)) or lacking Rro (E. coli (pAG58)). The samples were run through a non-denaturing 6% polyacrylamide gel. Lanes 1 and 4, no cell-free extract added; lanes 2 and 5, extract of E. coli (pAG58SR) added; lanes 3 and 6, E. coli (pAG58) extract added.

Expression of ORF5 is subject to repression by Rro

To study possible transcriptional regulation of the r1t region by Rro, a lacZ translational fusion was constructed with ORF5 in plasmid pIR12 (Fig. 7). In L. lactis LL302 carrying pIR12, very little β-galactosidase activity was observed (Fig. 8). To determine whether lacZ expression could be induced under conditions which normally induce the switch from the lysogenic to the lytic life cycle of r1t, the effect of mitomycin C on β-galactosidase activity was examined. After the addition of 1 µg ml⁻¹ of mitomycin C to an L. lactis LL302 culture carrying pIR12, β-galactosidase activity increased considerably. Two and a half hours after the addition of mitomycin C, β-galactosidase activity had increased approximately 70-fold, indicating that ORF5 expression is transcriptionally regulated, presumably by promoter P₂. In order to examine whether the low lacZ expression in non-induced cells carrying plasmid pIR12 was established through repression of (P₂) promoter activity by Rro, pIR13 was constructed.

Filling-in of the NcoI restriction-enzyme site of pIR12 resulted in a frameshift mutation and the introduction of two stop codons in rro. Therefore, cells containing pIR13 do not produce functional Rro. As can be seen in Fig. 8, such cells constitutively express lacZ at a high level. From these results we infer that the rro gene is required for repression of ORF5 transcription under conditions that favour the lysogenic state of the bacteriophage r1t.

Discussion

In this study we have identified a regulatory region of the temperate L. lactis bacteriophage r1t that is most probably involved in the control of the lysis-lysogeny 'decision'. On the basis of significant similarity of its deduced amino acid sequence with various repressor proteins, we assumed that rro specifies the bacteriophage r1t repressor protein. The rro gene is immediately preceded by promoter P₁. The identity of the -10 and -35 sequences of P₁ to the L. lactis vegetative promoter consensus is consistent with the fact that the lysogenic response to infection by a temperate phage requires the synthesis of a phage-encoded repressor (Ptashne, 1986). The repressor gene, therefore, is probably one of the first phage genes to be expressed after infection and, consequently, its expression should rely entirely upon phage-specific transcription initiation sequences recognized by the host RNA polymerase. The -35 and -10 sequences of P₂ are identical to those of P₁ but the presence in the latter of a -16 region in the spacer between both hexamers could account for possible bias in promoter utilization towards P₁. This 5'-RTRTG-3' sequence, positioned one base upstream of the -10 hexamer of P₁, appears to be conserved in many promoters of Gram-positive bacteria (Moran et al., 1982; van der Vossen et al., 1987). For B. subtilis, it has been shown that this motif positively affects both promoter strength and utilization (Voskuil et al., 1995).

On the basis of the N-terminal homology between Rro and the Tuc2009 CI repressor, we assume that the first possible initiation codon downstream of P₁ functions as the translation initiation signal of rro. As the adenine of this codon also functions as the transcription start site, this would be a situation analogous to that in the E. coli bacteriophages lambda and HK022. The Prm transcripts of lambda and HK022 initiate at the A of the AUG start codons of the repressor genes and, therefore, lack 5'-leader and Shine-Delgarno (SD) sequences (Ptashne et al., 1976; Cam et al., 1991). For the lambda CI transcript it has been shown that translation depends on signals different from the conventional ribosome binding site (RBS) (Shean and Gottesman, 1992).

On the basis of the observed homology with specific portions of other phage-encoded repressor proteins, Rro seems to have a two-domain structure: an N-terminal portion

© 1996 Blackwell Science Ltd, Molecular Microbiology, 19, 1331–1341
involved in binding to the operator sites, and a C-terminal domain which mediates dimer formation, dimer–dimer interaction, and self-cleavage. Upon DNA damage, self-cleavage of a phage repressor can occur via a RecA-mediated pathway which results in prophage induction. This autodigestion takes place in the so-called 'hinge region' connecting the N- and C-terminal domains at a specific site that is conserved in proteins that can undergo RecA-mediated cleavage (Little, 1993). These conserved amino acids are also present in the deduced amino acid sequence of Rro (Fig. 2). A putative DNA-binding motif is present in the N-terminal part of Rro. Like most of the bacteriophage-specified repressor proteins, Rro contains a so-called helix-turn-helix motif, suggesting that this stretch of amino acids is involved in binding to a specific DNA target, the operator (Pabo and Sauer, 1992). Despite
the high overall sequence similarity, the Rro and Tuc2009 CI repressor proteins differ in their N-terminal portions. It is therefore likely that both proteins recognize different DNA sequences.

Most of the bacteriophage operators described hitherto consist of imperfect symmetrical binding sites. In the case of phage lambda operators this twofold rotational symmetry reflects the two binding sites for the two monomers forming a repressor dimer (Pabo and Lewis, 1982). The subtle structural variation in the individual binding sites of the operators forms the basis for the differential relative affinities of CI and Cro for these sites (Ptashne, 1986). The three r1t operators differ slightly from each other and might function similarly. Indeed, Rro also binds O2 and O3, albeit with a lower affinity (results not shown). Of the 11 bp r1t consensus operator, seven nucleotides are invariant. Preliminary results indicated that Rro is unable to bind a 21 bp operator site of which one of these conserved nucleotides was substituted (results not shown).

The relative position of the putative operators of r1t clearly differs from that in bacteriophage lambda. Whereas in lambda all three operators are clustered in the non-coding area between cl and cro, enabling co-operative binding of repressor molecules, the centre of r1t O2 is located 402 bp upstream from that of O2. Such spacious arrangements of multiple operator sites have been demonstrated in the E. coli operons gal (Fritz et al., 1983; Irani et al., 1983), araBAD (Dunn et al., 1984), deo (Vallentin-Hansen et al., 1986) and lac (Eismann et al., 1987). In all these cases, there is now accumulating evidence for a regulatory mechanism that involves co-operative binding of repressor to the distant sites through protein–protein contacts holding together a loop of intervening DNA (Schleif, 1992; Matthews, 1992). A similar situation has also been demonstrated for the B. subtilis phage φ105 (van Kaer et al., 1987). Operator O2 of φ105 is located approximately 250 bp downstream of O3, within the proximal gene of the P2 transcription unit. Analogous to the regulatory regions of several other temperate bacteriophages, the region encompassing ro and tec is probably involved in the control of lysogeny of r1t. In this scheme, P1 is apparently responsible for the establishment of the lysogenic state, whereas P2 functions as the transcriptional start signal for the genes expressed during the lytic stage, being repressed during lysogenic growth. Indeed, expression of a lacZ fusion with ORF5, which is located downstream of P2, was very low under conditions that favour the lysogenic life cycle of r1t. Expression of the lacZ fusion could be induced by the addition of 1 µg ml⁻¹ mitomycin C, a concentration which in r1t promotes the switch to the lytic life cycle. Derepression of ORF5 transcription in non-induced cells in the absence of functional Rro (L. lactis (pIR13)) strongly suggests that Rro represses promoter P2, because the region between tec and ORF5 does not provide space for a promoter, and because no promoter-like sequence could be discerned within tec.

On the basis of the results presented here, it would appear that the general strategy employed by r1t to control lysogeny is similar to that used by the lambdoid phages of E. coli. In bacteriophage lambda, cro is the first gene that is transcribed upon induction. Cro prevents transcription of the genes expressed during the lysogenic life cycle by binding to the same operator sites that CI recognizes. The order in which Cro binds to these sites, however, is opposite to that of CI. Although tec is the topological equivalent of lambda cro, it could specify a protein of approximately the same size as Cro, and the deduced amino acid sequence contains a putative DNA-binding α-helix–turn–α-helix motif, it still has to be clarified whether the tec gene product actually directs the bacteriophage into the lytic cycle and, if so, whether it is the functional equivalent of cro.

This study showed that a regulatory region of the lactococcal bacteriophage r1t can be exploited for inducible gene expression in L. lactis. Inducible gene expression in E. coli based on the temperature-sensitive CI repressor mutant CI857 has been extremely helpful as a simple means by which to overexpress (heterologous) genes in this organism. By analogy, such a system for lactococci would be valuable to modulate gene expression in this industrially important organism. Experiments are currently

**Fig. 8.** Effect of mitomycin C on β-galactosidase activities measured in L. lactis LL302 carrying plasmid pIR12 (○) as a function of time. t0 is the time point at which mitomycin C was added at a final concentration of 1 µg ml⁻¹. The β-galactosidase activities measured in cells carrying pIR12 and pIR13 without mitomycin C addition, are represented by (□) and (□), respectively. The time scale is in hours before and after t0.
Table 1. Bacterial strains, plasmids and bacteriophage used in this study.

<table>
<thead>
<tr>
<th>Strain/Plasmid</th>
<th>Relevant features</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. lactis subsp. cremoris LL302</td>
<td>MG1363 carrying the pWV01 repA gene on the chromosome</td>
<td>Leenhouts and Venema (1993)</td>
</tr>
<tr>
<td>E. coli MC1000</td>
<td>araD139 ΔlacX74 Δ(ara leu)7697 galU galK strA</td>
<td>Casadaban and Cohen (1980)</td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC18</td>
<td>AmpR</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pAG58</td>
<td>AmpR, CmR</td>
<td>Jaacks et al. (1989)</td>
</tr>
<tr>
<td>pAG58SR</td>
<td>AmpR, CmR; pAG58 derivative</td>
<td>This work</td>
</tr>
<tr>
<td>pMG57</td>
<td>EmR; pMG57 derivative</td>
<td>This work</td>
</tr>
<tr>
<td>pPXR1</td>
<td>AmpR; pUC18 derivative carrying ro</td>
<td>This work</td>
</tr>
<tr>
<td>Bacteriophage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>r1</td>
<td>Type P335, small isometric temperate lactococcal phage, isolated from L. lactis subsp. cremoris R1</td>
<td>Lowrie (1974)</td>
</tr>
</tbody>
</table>

a. For a key to the numbering of these restriction enzyme sites, see Fig. 7A. EmR, AmpR, resistances to erythromycin and ampicillin, respectively.

A 2.2 kb Hindlll fragment of bacteriophage r1t DNA, encompassing ro, tec, and parts of ORF2 and ORF5, was subcloned into the unique Hindlll site of pUC18, resulting in the plasmid pHD. The 1428 bp PvuII(2)-XbaI(1) fragment of pHd was inserted into pUC18 restricted with Smal and XbaI (Fig. 7A). The resulting plasmid, pPXR1, was restricted with Accl and the 5'-protruding ends were blunted with Klenow enzyme. The linearized vector was subsequently digested with EcoRI and the fragment carrying ro and part of tec was ligated into EcoRI/Smal-digested pMG57, resulting in plasmid pIR11. To restore tec and to fuse ORF5 in frame to the lacZ gene of E. coli, the 482 bp XbaI(1)-XbaI(2) fragment of pHD was cloned into the unique XbaI site of pIR11, resulting in plasmid pIR12. The amino acid sequence at the fusion site between ORF5 and lacZ is shown in Fig. 7B. To introduce a frameshift mutation into ro, pIR12 was restricted with Ncol and the resulting 5'-sticky ends were filled in with Klenow enzyme. After self-ligation, an NsiI restriction site was created in the resulting plasmid pIR13, as was confirmed by digestion with this enzyme, pIR12 and derivatives were propagated in L. lactis LL302 in order to increase the copy number of the plasmids by providing RepA in trans.

For the construction of pAG58SR, ro was amplified using PCR. An SD sequence was introduced upstream of ro in order to optimize expression of this gene in E. coli. The primers used for amplification had the following nucleotide sequences: 5'-gggagaacctgtgaggaatgaaAGTAAAAATACGACTACCTGAAATG-3' (upper-case sequence corresponds to positions 2757 to 2731 of the r1t nucleotide sequence; EMBL/GenBank/DDJB Nucleotide Sequence Data Library Accession Number U38906), and 5'-atagccgcatgCTTTTAACGAGAATCA-3' (of the sites are underlined in the PCR primers), the PCR fragment was subcloned in HindIII/Spcl restricted pAG58, in 100 µg ml⁻¹. The chromogenic substrate Xgal (Sigma Chemical Co.) was added to plates at a final concentration of 40 µg ml⁻¹.

DNA techniques

Plasmid DNA was isolated essentially by the method of Birnboim and Doly (1979). Restriction enzymes, Klenow enzyme, and T4 DNA ligase were obtained from Boehringer Mannheim and used according to the instructions of the supplier. For nucleotide sequence analysis the dideoxynucleotide chain-termination method (Sanger et al., 1977) was used with [α-35S]-dATP (Amersham International) and the T7 sequencing kit (Pharmacia). Synthetic oligonucleotides were synthesized using an Applied Biosystems 381A DNA synthesizer. PCR was performed using Vent polymerase (New England Biolabs Inc.). Samples were heated to 94°C for 2 min, after which target DNA was amplified using a Thermocycler 60 PCR apparatus (BioMed) in 25 subsequent cycles under the following conditions: 94°C for 1 min; 50°C for 2 min; 73°C for 1 min. Helix-turn-helix motif predictions were performed according to Dodd and Egan (1990).

Plasmid constructions

A 2.2 kb Hindlll fragment of bacteriophage r1t DNA, encompassing ro, tec, and parts of ORF2 and ORF5, was subcloned into the unique Hindlll site of pUC18, resulting in the plasmid pHD. The 1428 bp PvuII(2)-XbaI(1) fragment of pHD was inserted into pUC18 restricted with Smal and XbaI (Fig. 7A). The resulting plasmid, pPXR1, was restricted with Accl and the 5'-protruding ends were blunted with Klenow enzyme. The linearized vector was subsequently digested with EcoRI and the fragment carrying ro and part of tec was ligated into EcoRI/Smal-digested pMG57, resulting in plasmid pIR11. To restore tec and to fuse ORF5 in frame to the lacZ gene of E. coli, the 482 bp XbaI(1)-XbaI(2) fragment of pHD was cloned into the unique XbaI site of pIR11, resulting in plasmid pIR12. The amino acid sequence at the fusion site between ORF5 and lacZ is shown in Fig. 7B. To introduce a frameshift mutation into ro, pIR12 was restricted with Ncol and the resulting 5'-sticky ends were filled in with Klenow enzyme. After self-ligation, an NsiI restriction site was created in the resulting plasmid pIR13, as was confirmed by digestion with this enzyme, pIR12 and derivatives were propagated in L. lactis LL302 in order to increase the copy number of the plasmids by providing RepA in trans.

Experimental procedures

Bacterial strains, phage, plasmids, and media

The bacterial strains, phage and plasmids used in this study are listed in Table 1. E. coli was grown in TY broth (Rottlander and Trautner, 1970) or on TY broth solidified with 1.5% agar. L. lactis was grown in glucose M17 broth (Terzaghi and Sandine, 1975), or on glucose M17 agar. Erythromycin was used at 100 µg ml⁻¹ and 5 µg ml⁻¹ for E. coli and L. lactis, respectively. Ampicillin was added at a final concentration of
Table 2. Oligonucleotides used for the construction of the 21 bp synthetic double-stranded DNA fragments.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Nucleotide sequence</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oα</td>
<td>5' AAC TAG CCA ATT TGT CAA GTT 3'</td>
<td>3227–3207</td>
</tr>
<tr>
<td>Oβ</td>
<td>5' AAC TGG ACA ATT TGG CTA GTT 3'</td>
<td>3207–3227</td>
</tr>
<tr>
<td>Cα</td>
<td>5' GTC AAT CTA TTC AAT ACT GAT 3'</td>
<td>2598–2578</td>
</tr>
<tr>
<td>Cβ</td>
<td>5' AAT AGT ATT GAA TAG ATT GAC 3'</td>
<td>2578–2596</td>
</tr>
</tbody>
</table>

a. The positions correspond to the nucleotide sequences of bacteriophage r11 (EMBL/GenBank DDBJ Nucleotide Sequence Data Library Accession Number U38906).

pAG58SR rro is under the control of the IPTG-inducible P_{rro} promoter. The nucleotide sequence of rro was verified by nucleotide sequencing.

**Transformation**

*E. coli* was used as cloning host and transformed using the method of Mandel and Higa (1970). Plasmids were introduced into *L. lactis* LL302 by electrotransformation (Holo and Nes, 1989) with the modifications suggested by Leenhouts and Venema (1993).

**IPTG- and mitomycin C induction**

Overnight cultures were diluted 100-fold in fresh glucose M17 medium (*L. lactis*) or TY medium supplemented with 0.5% glucose (*E. coli*) and grown until the cultures had reached an OD_{600} of 0.3 (*L. lactis*) or 0.5 (*E. coli*). Mitomycin C (Sigma Chemical Co.) was then added to the *L. lactis* culture at a final concentration of 1 µg ml⁻¹. IPTG was added to *E. coli* at a final concentration of 5 mM. Before the addition of IPTG, *E. coli* cells were collected by centrifugation and resuspended in an equal volume of TY medium without additional glucose.

**Assay of β-galactosidase activity**

Cells from 5 ml of culture were collected by centrifugation and resuspended in 1 ml of cold Z-buffer (Miller, 1972). Glass beads (0.1 mm in diameter) were added and the cells were disrupted at 4°C for 15 min using a 'Shake it, Baby' cell disrupter (Biospec Products). Glass beads and cell debris were removed by centrifugation for 5 min at 20000 × g. Lysates were stored at -80°C. Oligonucleotides that were used for the construction of the 21 bp synthetic double-stranded DNA fragments are indicated in Table 2. The single-stranded synthetic oligonucleotides Oα and Cα (30 ng) were end-labelled with polynucleotide kinase (Pharmacia) in the appropriate buffer for 1 h at 37°C using 30 µCi [γ-^{32}P]-ATP (Amersham). Labelled oligonucleotides were collected by Sephadex G-50 chromatography (Wall et al., 1988) and purified by phenol/chloroform extraction. After ethanol precipitation in the presence of 1 µg poly(dI–dC) and 1 mM MgCl₂, the labelled oligonucleotides were annealed with their 'cold' synthetic complementary DNA fragments, Oβ and Cβ (120 ng), in 1 × HIN (6 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 50 mM NaCl, and 1 mM dithiothreitol (DTT)) by heating for 2 min at 90°C, after which the samples were cooled to 4°C. Binding was carried out in 20 µl reaction volumes containing 20 mM Tris-HCl pH 8.0, 20% (v/v) glycerol, 1 mM EDTA pH 8.0, 200 mM KCl, 1 mM DTT, 100 µg ml⁻¹ acetylated bovine serum albumin, 100 µg ml⁻¹ poly(dI–dC), labelled DNA fragment (approximately 75 pg), and cell lysate (1–9 µg). After incubation for 15 min at room temperature, the samples were loaded onto a 6% polyacrylamide gel containing 3% glycerol. Gels were run in TAE buffer (0.04 M Tris-acetate pH 7.5, 2 mM EDTA) at 150 V for 1.5 h and dried. The gels were used for autoradiography at -70°C using Kodak XAR-5 film and intensifying screens.

**Primer extension analysis**

RNA was isolated from exponentially growing *L. lactis* cells carrying pIR12 or pIR13, as described by van Asseldonk et al. (1993). Synthesis of cDNA was performed using the Boehringer reverse transcription–PCR (RT–PCR) (AMV) Kit essentially as described by the supplier. The mRNA (2 µg) of both strains was reverse transcribed using 50 ng of the synthetic oligonucleotides RPE (5'-CTTCAACCATGGGACTTCTAACCCTTTTATTACCTAGGC-3'; positions 2613–2651 of r1t nucleotide sequence) or TPE (5'-CCTGAAGAGTCTAACTCATCTAGTGGC-3'; positions 3039–3008 of r1t), and 10 µCi [^{35}S]-dATP (Amersham). Reaction mixtures were incubated for 10 min at 48°C and 46°C, respectively, followed by another 10 min in the presence of 0.5 mM dATP, after which loading buffer was added. Internally labelled extended products were separated by electrophoresis on a 6% polyacrylamide urea gel and analysed by autoradiography.

**Acknowledgements**

We thank the Unilever Research Laboratory, Vlaardingen, for financial support; Dr A. M. Ledeboer, Dr W. Musters, L. Hamoen, and B. J. Hajema for helpful discussions; and H. Mulder for photography. J.K. is the recipient of a fellowship.
References


Sanger, F., Nicklen, S., and Coulson, A.R. (1977) DNA
sequencing with chain termination inhibitors. Proc Natl Acad Sci USA 74: 5463–5467.