Transcription of the \textit{trp} operon in \textit{Lactococcus lactis} is controlled by antitermination in the leader region

Hélène Frenkiel, Jacek Bardowski,† S. Dusko Ehrlich and Alain Chopin

The regulatory functions of the leader region preceding the \textit{Lactococcus lactis} \textit{trp} operon have been studied by mutagenesis analysis. This leader presents striking similarity to 'T-box' leaders found upstream of many Gram-positive aminoacyl-tRNA synthetase genes and some amino acid biosynthesis operons, which are controlled by antitermination through interaction of the leader transcript with cognate uncharged tRNA. A region of the \textit{L. lactis} leader transcript also contains a series of (GAU)AG repeats which, in \textit{Bacillus}, are involved in the binding of the \textit{trp} RNA-binding protein (TRAP) which controls \textit{trp} transcription. A screen was developed for the isolation of regulatory mutants affected in the leader region. All spontaneous mutants contained deletions; point mutations were only obtained after UV-induced mutagenesis. All mutations affected the putative transcription terminator upstream of the \textit{trp} operon, demonstrating that \textit{trp} is indeed controlled by transcription antitermination.

**Keywords:** \textit{Lactococcus lactis}, antitermination, tryptophan biosynthesis, \textit{trp} operon, transcription terminator

**INTRODUCTION**

All seven genes required for tryptophan (Trp) biosynthesis form an operon in \textit{Lactococcus lactis} (Bardowski \textit{et al.}, 1992), which is preceded by a 403-base non-coding leader region (Fig. 1). In the presence of Trp, two small transcripts are produced, corresponding to parts of the leader region: a 290-base transcript, initiated at the \textit{Ptrp} transcription promoter and arrested close to the putative transcription terminator T1 upstream of the structural genes, and a 160-base transcript, identical to the 3' part of the former, corresponding to a processing product (Raya \textit{et al.}, 1998). Under conditions of Trp starvation, transcription of a third additional transcript is induced 300- to 500-fold. This 8 kb transcript spans the full length of the operon.

The \textit{trp} leader region presents striking structural similarities to a family of leader regions upstream of aminoacyl-tRNA synthetase genes and some amino acid biosynthesis operons in a number of Gram-positive bacteria, which are controlled by antitermination (Grundy & Henkin, 1994). All these leader regions contain a transcription terminator upstream of the coding sequence. The sequence immediately upstream of the terminator, and extending within its 5' part, has the potential to form an alternative, but much less stable, antiterminator structure, in which a 14-base region, called the T-box, is highly conserved. The leader transcripts also have in common conserved stem–loop structures, a single codon, specifying the amino acid expected to regulate expression of the system, and an antiacceptor, complementary to the tRNA amino acid acceptor arm, present within the T-box (Fig. 1). The conservation of these features suggested a regulatory model in which transcription is controlled by a switch between the antiterminator and the terminator form of the leader transcript, mediated by interaction with the cognate uncharged tRNA (Grundy & Henkin, 1994). It is, however, likely that the regulatory mechanism is more complex, and that antitermination control involves additional interactions between the leader transcript and the cognate tRNA (Henkin, 1994; Gendron \textit{et al.}, 1994; Grundy \textit{et al.}, 1994; Putzer \textit{et al.},...
H. FRENKIEL and OTHERS

---

**Fig. 1.** Sequence and relevant features of the *trp* leader sequence. Numbers correspond to nucleotides in GenBank sequence M87483. –35, –10 and +1 refer to the consensus sequence of the *Ptrp* promoter, and to the transcription start, respectively. Boxed triplets indicate a region resembling the *Bacillus* TRAP-binding sites. Vertical arrows represent the site of processing of the leader (R. Raya and colleagues, unpublished results). RBS, ribosome-binding site.

1995; van de Guchte *et al.*, 1998). The possible involvement of regulatory protein factor(s) has also been considered (Grundy & Henkin, 1993; Grundy *et al.*, 1994).

It was observed by Bardowski *et al.* (1992) that the lactococcal *trp* leader contains two 10-base boxes, resembling the *Bacillus subtilis* and *B. pumilus* 'MtrB' boxes that are necessary for *trp* regulation in these bacteria. Further detailed studies on the molecular mechanism of this regulation revealed that *trp* antitermination in *Bacillus* is controlled by binding of a complex of 11 subunits of the *trp* RNA-binding protein (TRAP), which is encoded by the *mtrB* gene (Gollnick, 1994; Antson *et al.*, 1995). Binding occurs at a series of 11 closely spaced (G/U)AG repeats (Babitzke *et al.*, 1994, 1995). Examination of the lactococcal *trp* leader sequence revealed the existence of similar repeats (Fig. 1). This left open the possibility that the Trp-dependent binding of a lactococcal TRAP homologue participates in the *trp* control, since, in agreement with the model of tRNA-mediated antitermination, binding of a protein to the leader transcript would prevent formation of an antitermination structure, and lead to transcription termination.

To further characterize the tRNA*Trp*-mediated control of *trp* transcription and to identify other potential regulatory mechanism(s), we performed a mutagenesis analysis of the regulatory function of the *L. lactis trp* leader. This showed that the T1 transcription terminator is crucial for the regulation. No evidence was obtained that any other leader sequence is necessary for the repression of *trp* transcription in the presence of Trp.

**METHODS**

**Bacterial strains and transformation.** *Lactococcus lactis* IL1403 (Chopin *et al.*, 1984) and its derivatives, and *Escherichia coli* TG1 (Gibson, 1984), were grown as described by Bardowski *et al.* (1992). The chemically defined medium (CDM) for *L. lactis* has been described (Raya *et al.*, 1998).

**DNA and RNA manipulations.** Plasmid DNA was extracted as previously described (Bardowski *et al.*, 1992). *E. coli* cells were transformed according to the standard procedure using CaCl₂ (Sambrook *et al.*, 1989). *L. lactis* was transformed by an electroporation technique (Holo & Nes, 1989). RNA was
Strain constructions. A trpE-lacZ transcriptional fusion was constructed by inserting a 702 bp Sepl-Scal DNA fragment at the Smal site of plasmid pMC1871 (Sharpira et al., 1983). This fragment was recovered from plasmid pIL499 (Bardowski et al., 1992), and encompasses the entire trp leader and the first 84 codons of trpE. A transformant containing the insert, cloned in the proper orientation into pMC1871, was identified by its blue color on X-Gal-containing plates. The plasmid was designated pIL1130, and rendered integrative by formation of a co-integrate with pE194 (Horniouchi & Weisblum, 1982) at their unique PstI site. Plasmid pE194 contains an erythromycin-resistance determinant and is apparently unable to replicate in L. lactis IL1403, where it has already been used as delivery plasmid for chromosomal integration by homologous recombination (Chopin et al., 1989). This plasmid was designated pIL1131 and used to transform strain IL1403. One of the rare Ery<sup>R</sup> transformants was characterized by Southern blot analysis of its chromosomal DNA and shown to contain a single copy of pIL1131, inserted upstream of the trp operon. It was designated IL3655. Its β-galactosidase activity was 150-fold higher when cells were grown in CDM lacking Trp, as compared to cells grown on Trp-containing CDM, indicating that the expression of the trpE-lacZ fusion is controlled by Trp availability. Derivatives of IL3655 in which the trpE-lacZ fusion had been excised by homologous recombination were obtained as follows. Cells were grown for 30 generations in the absence of erythromycin, and plated on CDM + X-Gal without Trp. On this medium, cells containing the fusion form blue colonies. The rare white colonies, formed on this medium, were tested for sensitivity to erythromycin. Ery<sup>R</sup> clones were isolated at a frequency of 6 × 10<sup>-4</sup>, which is comparable with the frequency of recombination measured in L. lactis between homologous repeats of this length (Biswas et al., 1993).

Oligonucleotides. The following oligonucleotides were used: 1, 5'-GAGCCCTTCAAGAAACAATC-3'; 2, 5'-CCGTTACGC-3'; 3, 5'-CAAGGAAACATGTCACGGTGT-3'; 4, 5'-TGTAAAACGACGGCCAGTCGGGGTCA-3'; 5, 5'-CGGGATCCGGATCAGTGACGTTGTCAC-3'; 6, 5'-GGGATCCGGATCAGTGACGTTGTCAC-3'; 7, 5'-CGGGATCCGGATCAGTGACGTTGTCAC-3'; 8, 5'-GGGATCCGGATCAGTGACGTTGTCAC-3'. They were then amplified from their respective plasmids by PCR using oligonucleotide pairs 1-2 for strain IL3655, or 7-8 for strain IL56013, in a cycle extension reaction with dye-labelled terminators.

RESULTS

Development of a screen for trp regulatory mutants

A screen based on the use of a toxic tryptophan analogue was devised for the isolation of trp regulatory mutants. A Trp analogue, interacting with Trp regulation, would repress Trp biosynthesis. Only cells in which Trp biosynthesis is constitutive, e.g. regulation mutants, will grow in presence of this analogue. Nine Trp analogues were tested for their toxicity to L. lactis IL1403. Five were not toxic (5-methyltryptophan, 6-methyltryptophan, 3,β-indoleacyclic acid, 7-azatryptophan and indole-3-propionic acid), whereas in the presence of the others (4-fluorotryptophan, 5-fluorotryptophan, 6-fluorotryptophan or 4-methyltryptophan) plate counts were reduced by 4-6 orders of magnitude. This toxicity could result either from an analogue-dependent repression of Trp biosynthesis, or from the incorporation of the analogue into non-functional proteins. To differentiate the two possibilities, we reasoned that introduction of the trp leader carrier on a high-number-copy plasmid would titrate potential regulator(s), disturbing regulation of Trp biosynthesis, and thereby alleviating the toxic effect of the analogue. The trp leader and flanking regions were cloned into the high-copy-number plasmid pIL253 (Simon & Chopin, 1988), giving pIL1129. Among the four toxic analogues, only 6-fluorotryptophan (6-fTrp) was no longer toxic when IL1403 carried pIL1129. This suggested that 6-fTrp interferes with some trp regulator(s), and that screening for 6-fTrp resistance could be used to select for deregulated trp mutants.

To distinguish between mutants affected in the leader sequence, and those affected elsewhere, an IL1403 derivative designated IL3655 was used. This strain contains two copies of the trp leader, the first controlling the expression of the trp operon, and the second the expression of a trpE-lacZ fusion. Mutations in the leader, upstream of trp, rendering cells 6-fTrp<sup>R</sup>, would not affect the Trp-dependent response of the trpE-lacZ fusion. By contrast, mutations in trans conferring 6-fTrp<sup>R</sup> would be likely also to affect the Trp-dependent response of the trpE-lacZ fusion. The two classes of events should therefore be distinguishable by the colony colour on plates of CDM containing X-Gal, 6-fTrp and 1 and 2 were used to amplify the leader region from strain IL3655, and oligonucleotides 7 and 8 were used to amplify the same region from strain IL56013. The sequence of the PCR products was determined, using one of two alternative strategies. (1) Overlapping subfragments were amplified by PCR using oligonucleotide pairs 3-4 and 5-6. These oligonucleotides contain tails complementary to either the 21M13 or M13 reverse sequence. The sequence of the PCR products was determined in a cycle extension reaction with Taq DNA polymerase and fluorescent dye-coupled -21M13 or M13 reverse primers, on a 373 DNA sequencer (Applied Biosystems). (2) The sequence of the PCR fragment was directly determined, using oligonucleotide pair 1-2 for strain IL3655, or 7-8 for strain IL56013, in a cycle extension reaction with dye-labelled terminators.

Vegetative growth. Cells in the mid-exponential phase of growth were washed in Kinger's solution and diluted were plated on M17 glu agar (Terzaghi & Sandine, 1975) or on CDM without tryptophan supplemented with 40 μg X-Gal ml<sup>-1</sup>, 5 μg erythromycin ml<sup>-1</sup> and 100 μg 6-fluorotryptophan (6-fTrp) ml<sup>-1</sup>. Dried plates were irradiated with the appropriate dose of 254 nm UV light, using a Stratalinker UV cross-linker (Stratagene). Colonies were counted after 48 h incubation at 30 °C, and the frequency of mutation was calculated as the ratio of the number of 6-fTrp<sup>R</sup> mutants to the number of c.f.u. on M17.

Characterization of the mutants. DNA fragments containing the mutant trp leader were produced by PCR amplification, using appropriate oligonucleotides. Oligonucleotides extracted from L. lactis, analysed by Northern experiments, and quantified as described by Raya et al. (1998). Other molecular techniques were carried out using established procedures (Sambrook et al., 1989).
no Trp. Mutants in cis will form white colonies, whereas mutants in trans will form blue colonies.

**Selection and characterization of spontaneous trp regulatory mutants**

Cultures of IL3655 were plated on CDM lacking Trp, supplemented with 6-fTrp, erythromycin and X-Gal. Cells formed colonies at a mean frequency of $2 \times 10^{-6}$, and 25% of them were white. Sixty-eight white colonies were picked from independent cultures and the mutations were characterized by sequencing of their leader region, as described in Methods. Sixty-six mutants contained a deletion in the leader sequence, affecting all or part of the putative transcription terminator sequence (Fig. 2). The deletions fell into 17 classes, one containing the majority of the mutations. Interestingly, while the vast majority of the mutations had the potential to drastically affect the structure of the terminator, two (IL6005 and IL6029) introduced much more limited changes, still leaving the potential of forming a stem-loop structure, followed by a run of uracil residues. In mutant IL6030, the stem-loop sequence is left intact, and the deletion removed the immediately downstream run of uracil residues.

Two mutants were not affected in the sequence of the transcription terminator. Mutant IL6040 contained a G-728-T transversion. This mutation affected one of the (G/U)AG repeats which form a sequence resembling the *B. subtilis* TRAP-binding site (Fig. 1). Mutant IL6038 exhibited no change in its trp leader sequence. Further analysis of this mutant revealed that the plasmid integrated into its chromosome had been amplified by homologous recombination, a phenomenon already observed in *L. lactis* (Chopin et al., 1989). The role of this amplification on the resistance to 6-fTrp was established by showing that an IL6038 derivative in which the amplified structure has been deleted by homologous recombination (see Methods), was no longer resistant to 6-fTrp. To avoid the isolation of such mutants, strain IL56013 (van de Guchte et al., 1998), which contains the trpE-lac2 fusion inserted at the his locus by double crossing-over, and is not likely to be prone to amplification through recombination, was used in subsequent experiments.

All but one of the 6-fTrpR mutants in cis are affected in the sequence of the putative transcription terminator, indicating that this sequence is a key element in the control of the trp operon. These spontaneous deletion mutants were most probably produced by an illegitimate recombination event, since 89% of the deletions were located between direct repeats with a length between 3 and 10 bp. The isolation of a point mutation, mapping outside this region, suggested the existence of another level of control and prompted us to isolate more mutants of this latter type. To isolate mutants avoiding the observed large prevalence of deletions, we attempted to increase the frequency of point mutations by using UV irradiation.

**Characterization of UV-induced 6-fTrpR mutants**

6-fTrpR mutants of IL56013 were selected after irradiation with 20 J m$^{-2}$ of UV light. Cells formed colonies at a mean frequency of $2.5 \times 10^{-6}$, and 7% of them were white. Forty-two white colonies were picked
from independent cultures. Mutants in which a deletion in the trp leader region had occurred were identified by the decreased length of the PCR product obtained from their DNA, using oligonucleotides 7 and 8, as compared to that of the wild-type PCR product. This revealed that 37 (88%) of the mutants carried a deletion in the leader, and the sequence of the five remaining mutants was determined (Fig. 3). Mutants IL6164 and IL6167 carried a duplication of 8 bp and 23 bp, respectively, internal to the terminator sequence. Mutant IL6166 carried a tandem double substitution located in the terminator stem region. Mutant IL6165 was similar to IL6166, except that it carried an additional, closely spaced, single substitution.

In this experiment, despite a slight increase in the frequency of non-deletion mutants, no point mutation mapping outside the terminator was induced. We therefore tried to further increase the point mutation frequency by using a UVR determinant, encoded by the lactococcal plasmid pIL7 (Chopin et al., 1985). This determinant has been cloned as a 5.5 kb DNA fragment in the pHV1301 vector plasmid, giving pIL31 (Chopin et al., 1986). We reasoned from previous observations (M.-C. Chopin, unpublished results), that this UVR determinant was likely to function through an error-prone repair mechanism and could therefore increase the mutation frequency after UV irradiation. An IL6159(pIL31) derivative, designated IL6159, was therefore used in UV mutagenesis.

Cells of IL6159 were irradiated with 50 J m\(^{-2}\) of UV light. Colonies appeared at a mean frequency of 10\(^{-4}\) and 5% were white. Ten out of 22 mutants studied carried a deletion in the leader, and 12 carried point mutations, which fell into nine classes (Fig. 3). As expected, the frequency of point mutations was strongly increased in the presence of pIL31, but all mutations identified still affected the sequence of the transcription terminator.

A few UV-induced 6-fTrp\(^{R}\) mutants were not affected in the sequence of their trp leader, suggesting that they carried a mutation in trans which was not identified by the screen used. This was confirmed by careful comparison of the colour of the colonies of the mutants and the parent, which formed very pale blue and white colonies, respectively, on X-Gal-containing plates. It is likely that in these mutants, a mutation in trans slightly disturbed the regulation of both the trp operon and the trpE-lacZ fusion, leading to a barely scorable blue colour of the colony and incorrect assignment of the mutation. Its effect was, however, sufficient to confer resistance to 6-fTrp.

Transcriptional analysis of the mutants

Transcription of the trp operon, in the presence or absence of Trp, was compared in Northern experiments, in the wild-type strain IL1403, and in two regulatory mutants. The presence in these mutants of two copies of

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutagenesis</th>
<th>Number of occurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL1403</td>
<td>Spontaneous</td>
<td>1</td>
</tr>
<tr>
<td>IL6005</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6029</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>IL6030</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6007</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6164</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6165</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>IL6166</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6167</td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>IL6168</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6169</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6170</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6171</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6172</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6173</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6174</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6175</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>IL6176</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Fig. 2. Characterization of the 6-fTrp\(^{R}\) mutants. Mutants IL6005 to IL6007 are derivatives of IL3655. Mutants IL6164 to IL6167 are derivatives of IL56013. Mutants IL6168 to IL6176 are derivatives of IL6159 (IL56013 containing the UVR plasmid pIL31: Chopin et al., 1986). All mutants were affected in the sequence of the transcription terminator. Changed nucleotides are indicated. Δ represents deletion of one nucleotide and the arrows indicate the stem of the terminator.
the *trp* leader might affect the regulatory function by titrating regulatory elements, and would prevent the individual quantification of the 290-base and the 160-base transcripts produced from the mutated leader. Therefore, the integrated plasmid was deleted by homologous recombination. Derivatives obtained may have recovered, upstream of the *trp* operon, either the wild-type *trp* leader, or the mutated copy. To differentiate between the two possibilities, the 6-fTrpR resistance of the derivatives was checked, and the sequence of the *trp* leader was verified.

Three mutants were successfully cured of their integrated plasmid, among which IL6103 and IL6182 carried the mutated *trp* leader sequence, as expected. In contrast, IL6104 had recovered the wild-type leader sequence. This unexpected result indicated that the G-728-T transversion was not responsible for the 6-fTrpR phenotype, which was most probably due to a second mutation in trans, as described above.

The three transcripts already observed (Raya et al., 1998) were found in IL1403 (Fig. 4): an 8 kb *trp*EGDCFBA transcript, and two small transcripts of 290 and 160 bases. In IL6182, which carries a deletion of nucleotides 818–843, removing more than half of the sequence able to form the terminator T1 stem, together with the six uracil residues present immediately downstream, only the 8 kb transcript was visible. The absence of the 290-base and 160-base transcripts confirmed that they are arrested at T1 in IL1403, and indicates that the T1 terminator function is fully abolished in IL6182. In the same mutant, the 8 kb transcript was still produced in the presence of an excess of Trp, demonstrating that T1 is involved in the Trp-dependent control of *trp* transcription.

Transcription in IL6103, which is affected by a short deletion of nucleotides 834–835 in the T1 sequence, was very similar to that in IL1403. The 290-base and 160-base transcripts were present, and the production of the 8 kb transcript seemed to be efficiently controlled by Trp availability. This indicates that the terminator function in this mutant is only slightly affected by the deletion. The observation that this mutation, however, confers 6-fTrpR upon IL6103 confirms that a slight deregulation of *trp* transcription is sufficient to confer 6-fTrpR.

**DISCUSSION**

A mutagenesis approach was used to identify regions of the lactococcal *trp* leader which are necessary to repress transcription of the *trp* operon in the presence of tryptophan. A screen was developed, based on the acquisition of resistance to the toxic Trp analogue 6-fTrp, which allowed isolation of deregulated *trp* mutants. All mutants characterized in this study were affected in the sequence of the transcription terminator T1, upstream of the *trp* operon. This demonstrated the importance of this sequence for *trp* termination. Study of transcription in one of these mutants, IL6182, which lacks more than half of the sequence able to form the terminator T1 stem, together with the immediately downstream uracil residues, revealed that terminator function is fully abolished in this mutant, and demonstrated the terminator function of T1.

The putative transcription terminator T1, upstream of the *trp* operon, was identified by Bardowski et al. (1992), who noted its high structural similarity to *E. coli* intrinsic terminators. Its functionality has been inferred by R. Raya and colleagues (unpublished results), who showed that an 86 bp DNA segment containing T1 had transcription-terminator activity. In this study, we identified mutations affecting T1 activity, demonstrating that T1 possesses typical functional features of prokaryotic intrinsic transcription terminators. In addition to the many mutants in which all or most of the T1 sequence has been deleted, some contained more limited changes, allowing some structure–function analysis of the terminator to be made.

A first class of mutants contained short deletions or point mutations affecting the T1 stem. All are located within the GC-rich region, near the base of the stem, which is highly conserved in most *E. coli* terminators (d'Aubenton Carafa et al., 1990) and is important for terminator function (Lynn et al., 1988; Cheng et al.,
One of these mutations, in IL6103, had no measurable effect on the terminator function. The deletion is expected to produce a stem with a 2-base bulge, a feature which is sometimes present in spontaneous functional terminators (d’Aubenton CaraFA et al., 1990). In other terminators, changes in the stem sequence had a limited effect on terminator efficiency (Lynn et al., 1988; Cheng et al., 1991; Wilson & von Hippel, 1995).

A second class of mutations left the stem intact, affecting either the run of uracil residues immediately downstream, or the length of the loop. In IL6030, the six consecutive uracil residues were deleted, together with the 8 nt downstream. In this mutant, the T1 stem is followed by a run of only two uracil residues, a length which has been shown to be insufficient to produce termination (Lynn et al., 1988). Termination efficiency could not be measured in IL6030, since we did not succeed in obtaining a derivative from which the integrated plasmid pIL1131 was excised. However, the 6-\textit{fTrp}\textsuperscript{R} phenotype of this mutant indicates that the mutation has probably affected transcription efficiency. Mutant IL6167 contained a duplication of 23 bp which should produce two consecutive stem–loop structures, the last one separated by one cytosine from the run of uracil residues. Insertion of a non-uracil nucleotide between the stem and the run of uracil residues has not been found among known functional transcription terminators (d’Aubenton CaraFA et al., 1990). Mutant IL6164 contained a duplication of 8 bp, resulting in an increased length of the loop, which affected transcription efficiency as revealed by the conferred 6-\textit{fTrp}\textsuperscript{R} phenotype. This effect of an increased loop size on termination efficiency has apparently not been reported before. However, decreasing the size of a terminator loop from 8 to 4 bases slightly increased its efficiency (Wilson & von Hippel, 1995). Moreover, it was observed that 85\% of the \textit{E. coli} transcription terminators have loops 3–5 nt long, suggesting an optimal size for terminator function (d’Aubenton CaraFA et al., 1990).

Almost all the spontaneous 6-\textit{fTrp}\textsuperscript{R} mutants \textit{in cis} obtained in this work carried deletions. Short direct repeats (3–10 bp long) were present at the ends of most of the deletions, suggesting that they had occurred through illegitimate recombination (Ehrlich, 1989). The most frequent deletions (56\% of the total), occurred between the longest direct repeats present in the \textit{trp} leader (10 bp). The fact that all the mutants carried deletions contrasts with the observation that illegitimate recombination is generally rare in bacteria, relative to point mutation (Shapiro, 1985; Ehrlich, 1989).

The use of UV irradiation, alone or coupled to the UV-resistance determinant carried on plasmid pIL31 (Chopin et al., 1983, 1986), allowed us to increase the relative frequency of point mutations, especially in the presence of pIL31. UV irradiation alone led to the isolation of two point mutations, whereas UV irradiation in the presence of pIL31 led to the isolation of 12 point mutations (representing 5 and 55\%, respectively, of the number of mutants obtained). The spectrum of mutations observed after UV irradiation was very different from what has been observed in \textit{E. coli} (Wood & Hutchinson, 1987; Livneh et al., 1993). The most frequent changes in our mutants were complex changes, involving tandem double substitutions (64\%) and tandem double substitutions associated with a single substitution nearby (14\%). Single substitutions and frameshifts were rare, in contrast to what has been observed in \textit{E. coli}, where they are the most frequent UV-induced changes, representing between 95 and 97\% of the mutations observed in the \textit{lacD} and \textit{cl} genes (Wood & Hutchinson, 1987; Livneh et al., 1993). This strong contrast between the mutational spectra observed in \textit{L. lactis} and \textit{E. coli}, in both spontaneous and UV-induced mutants, may reflect either the existence of a peculiar mutagenesis system in \textit{L. lactis}, or a specific bias in the selection of 6-\textit{fTrp}\textsuperscript{R} mutants affected in their \textit{trp} leader. In spite of the fact that few lactococcal mutations have been characterized, the available evidence indicates that single substitutions are not uncommon in \textit{Lactococcus} (Dickely et al., 1995; Goupil et al., 1996), suggesting that the unusual mutational spectra observed are, rather, due to a specific selective bias linked to our target DNA. All the point mutations but one, affecting the T1 transcription terminator, were complex events, involving several nucleotides. Since single substitutions may occur in \textit{L. lactis}, this result is most probably due to the fact that only a few rare single substitutions (like the one in IL6174) are able to affect the terminator function and that more extended lesions are in general necessary. This is further substantiated by the observation that a double tandem substitution in the T1 stem (IL6103) only slightly affected termination efficiency.

\textit{trp} transcription was not measurably affected in mutants IL6103 or IL6104, as compared to transcription in the wild-type parent. A weak deregulation of Trp biosynthesis is therefore sufficient to confer resistance to 6-\textit{fTrp}, indicating that the use of this screen must have allowed the isolation of mutants weakly affected in the regulation.

No leader mutation conferring 6-\textit{fTrp}\textsuperscript{R} has been obtained outside the T1 sequence. This may reflect functional constraints: point mutations could affect higher-order structure of the leader, and hence, its antitermination function. Such mutants would not be selected in our system. This hypothesis, based on a strong conservation of the leader sequence, was ruled out by comparing sequence conservation of the \textit{trp} leader, and the chromosomal \textit{ldh} gene, between \textit{L. lactis} IL1403 and MG1363 (Gasson, 1983). The \textit{trp} leader sequence of MG1363 was determined (not shown). Sequence conservation between the two strains was 96-6\% in the \textit{trp} leader and 96-4\% in the \textit{ldh} gene (Swindell et al., 1994), indicating that the leader sequence has not been particularly conserved during evolution. The absence of mutations outside the T1 sequence therefore constitutes indirect evidence that no evolution, other than the transcription terminator, is involved in repression of \textit{trp} by Trp.
ACKNOWLEDGEMENTS

We gratefully acknowledge Costa Anagnostopoulou, Marie-Christine Chopin and Maarten Van de Guchte for their kind help in the preparation of the manuscript. This work was supported by contract Bridge-Biot-CT91-0263 of the Commission of the European Communities.

REFERENCES


family and its use for molecular cloning in *Streptococcus lactis.* Biochimie 70, 559–566.


