

## Efficiency of Transcription from Promoter Sequence Variants in *Lactobacillus* Is Both Strain and Context Dependent

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**The introduction of consensus –35 (TTGACA) and –10 (TATAAT) hexamers and a TG motif into the *Lactobacillus acidophilus* ATCC 4356 wild-type *slpA* promoter resulted in significant improvements (4.3-, 4.1-, and 10.7-fold, respectively) in transcriptional activity in *Lactobacillus fermentum* BR11. In contrast, the same changes resulted in decreased transcription in *Lactobacillus rhamnosus* GG. The TG motif was shown to be important in the context of weak –35 and –10 hexamers (*L. fermentum* BR11) or a consensus –10 hexamer (*L. rhamnosus* GG). Thus, both strain- and context-dependent effects are critical factors influencing transcription in *Lactobacillus*.**

The bacterial RNA polymerase (RNAP) major  $\sigma$  factor recognizes and binds to conserved hexamers located at positions –35 (TTGACA) and –10 (TATAAT) with respect to the transcription initiation site in most organisms examined to date. These promoter hexamers are most frequently separated by a spacer of  $17 \pm 1$  nucleotides, which often harbors a TG motif upstream of the –10 hexamer. Mutations which result in increased identity to the consensus hexamers generally lead to an increase in promoter efficiency in both gram-negative (11, 13, 20) and gram-positive (4, 10, 19) bacteria. The presence of the TG motif appears to be of considerable importance in gram-positive organisms, where introduction or deletion of the motif can influence promoter activity substantially (9, 18, 19).

While extensive studies of promoter sequences in *Escherichia coli* have been conducted (5, 11), promoters of gram-positive bacteria, with the possible exception of *Bacillus subtilis*, remain largely uncharacterized. Indeed, mutational analysis of promoters in the industrially and medically important gram-positive organism *Lactobacillus* has not been previously reported. Surveys of *Lactobacillus* promoters have revealed significant conservation of bases in the –35 and –10 regions, with consensus sequences identical to those of other organisms (11a, 15). The TG motif is a conserved feature in 26% of all *Lactobacillus* promoters, with the conservation frequency varying markedly between species (11a). Data suggest that the –35 hexamer, –10 hexamer, and TG motifs are significant determinants of promoter strength in lactobacilli (11a). The potential exists, therefore, to improve expression of heterologous genes in lactobacilli by specifically targeting these core promoter elements to enhance transcription initiation.

In the present study, we introduced consensus –35 and –10 hexamer elements and a TG motif into the wild-type *Lactobacillus acidophilus* ATCC 4356 *slpA* promoter (2) and examined the effects on promoter activity in two strains of *Lactobacillus*, *L. fermentum* BR11 and *L. rhamnosus* GG.

The *slpA* wild-type promoter region (extending from posi-

tion –286 to –20, with respect to the translation start codon [2]) was initially amplified from *L. acidophilus* ATCC 4356 genomic DNA by using the primer pair 5'-AAAAGGATCCTGCTTGTTGGGGTAAGCGG-3' and 5'-TTTTCTGCAGATA TAAAAAATGTAATAGGCC-3'. The promoter fragment was introduced into the *Bam*HI/*Pst*I sites of the multiple cloning site of plasmid pNZ272<sup>RBS</sup>, a ribosome binding site-deficient derivative of the *E. coli*-*Lactobacillus* shuttle vector pNZ272 harboring the promoterless *gusA* reporter gene (14). The use of this plasmid, which carries defective *gusA* protein machinery, avoids the deleterious effects associated with expression of the  $\beta$ -glucuronidase enzyme in these strains of lactobacilli (11a). Seven *slpA* promoter derivatives (Table 1) were generated by ligation of a 5'-phosphorylated oligonucleotide harboring the mutant base(s) during PCR of the *slpA* fragment (primer sequences and templates used are available upon request). PCR and cycling conditions were as previously described (12), with the following modifications: 1.25 mM MgCl<sub>2</sub>, 50 pmol of each primer, 200  $\mu$ M (each) deoxynucleoside triphosphate, 1 to 15 ng of template (genomic or plasmid) DNA, 1 to 3 U of Expand High Fidelity *Taq* DNA polymerase (Boehringer Mannheim), and a 55°C annealing temperature. The *slpA* derivative PCR products were purified, digested with *Bam*HI/*Pst*I, ligated to *Bam*HI/*Pst*I-digested pNZ272<sup>RBS</sup>, and transformed into *E. coli* KW1 for selection of mutant clones. Plasmid constructs were sequenced to screen for incorporation of the desired base substitutions. Positive constructs were transformed into *L. fermentum* BR11 and *L. rhamnosus* GG essentially as previously described (16). Penicillin concentrations of 1 and 10  $\mu$ g/ml were used for *L. fermentum* BR11 and *L. rhamnosus* GG, respectively. Plasmid constructs from lactobacilli were analyzed by restriction digestion and direct sequencing to ensure plasmid integrity and maintenance of the mutant bases within the *slpA* promoter sequence.

The activity of each promoter in lactobacilli was determined directly by a quantitative Northern blot procedure. Total RNA was extracted from *Lactobacillus* clones (14), electrophoresed through 1.5% agarose formaldehyde gels, and transferred to positively charged nylon membranes (Boehringer Mannheim) by capillary transfer (17). Membranes were subsequently probed with a digoxigenin (DIG)-labelled 895-bp *gusA* PCR fragment (produced from linearized pNZ272<sup>RBS</sup> with the primer pair 5'-AATGTCACCTAACCTGCC-3' and 5'-GGGTTGGGGTTTCTACAGGACGTA-3') to determine specific

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TABLE 1. *slpA* promoter base substitution mutants and relative transcriptional activities in *Lactobacillus* strains

Plasmid	Sequence	Relative <i>gusA</i> transcript level <sup>a</sup>	
		<i>L. fermentum</i> BR11	<i>L. rhamnosus</i> GG
pNZslp <sup>b</sup>	-35 TG -10 TTGCTATTTCTTGAAGAGGTAGTACAAT	100	100 (450)
pNZS10	-----T-----	412*	7.5** (34)
pNZS35	---AC-----	426**	33** (149)
pNZS1035	---AC-----T---	312	100 (450)
pNZTG	-----G-----	<b>1,072**</b>	15** (68)
pNZT10	-----G---T---	<b>500**</b>	16** (72)
pNZT35	---AC-----G---	116	16** (72)
pNZT1035	---AC-----G---T---	131	212** ( <b>954</b> )

<sup>a</sup> Represented as percent wild-type activity (values in parentheses represent *L. rhamnosus* GG transcript levels normalized to those of *L. fermentum* BR11). Significance levels (Student's *t* test) are as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . Values in boldface type are maximal *gusA* mRNA levels in each strain.

<sup>b</sup> Wild type.

transcript levels and with a DIG-labelled species-specific 212-bp 16S rRNA PCR fragment (produced from genomic DNA with primer pair 5'-AGCAGCCGCGTAATAC-3' and 5'-CTTGCGCACTGGGTGTTC-3') to standardize for gel loading and transfer. Hybridization signals were quantified via highly standardized optical scanning (Scanjet 4C; Hewlett Packard) and densitometry (Imagequant; Molecular Dynamics). Relative transcript levels were corrected for relative plasmid copy numbers (14) within each recombinant strain to eliminate gene dosage effects. Student's *t* test was used to define significant differences between means of at least three independent determinations for each *Lactobacillus* clone. Primer extension analysis of transcripts was done essentially as described by Platteeuw et al. (14), except that oligonucleotide GUS-AS was labelled with [ $\gamma$ -<sup>32</sup>P]ATP via T4 polynucleotide kinase (Boehringer Mannheim) prior to the annealing reaction. Transcription was initiated from the start site published for the natural host *L. acidophilus* ATCC 4356 (2) in both *L. fermentum* BR11 and *L. rhamnosus* GG (Fig. 1). The *slpA* wild-type promoter was utilized with 4.5-fold-higher efficiency in *L. rhamnosus* GG than in *L. fermentum* BR11. Mutants analyzed by primer extension indicated that the transcription start site remained unchanged following substitution of bases within the promoter region (data not shown). The *slpA* promoter is not known to be subject to regulation in vivo. The second promoter structure postulated by Boot et al. (3), located upstream of the *slpA* promoter, was not active in either *Lactobacillus* strain exam-

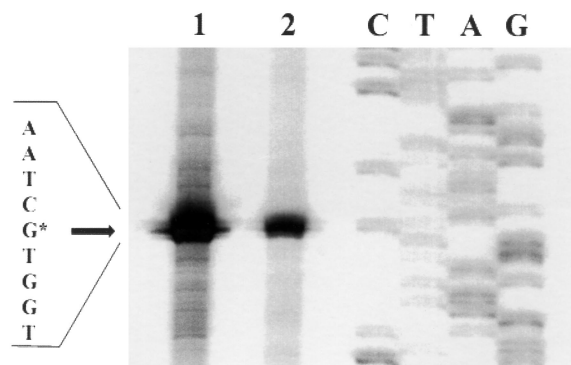


FIG. 1. Primer extension analysis of lactobacilli harboring the wild-type *slpA* promoter. Lane 1, *L. rhamnosus* GG; lane 2, *L. fermentum* BR11; lanes C, T, A, and G, sequencing reactions. Transcription was initiated from the start site published for the natural host *L. acidophilus* ATCC 4356 (2), with 4.5-fold-higher efficiency in *L. rhamnosus* GG than in *L. fermentum* BR11.

ined in the present study (data not shown). Thus, the changes in transcript levels described in this work were deemed to be due to the direct influence of the *slpA* promoter sequence on transcription initiation.

The effect of introducing consensus -35 and -10 hexamers and a TG motif into the *slpA* promoter was clearly dependent on the *Lactobacillus* strain in which it was harbored and varied when the promoter context was changed by the presence of another mutation (Fig. 2, Table 1). In *L. fermentum* BR11, introduction of an individual consensus -35 hexamer, -10 hexamer, or TG motif into an otherwise wild-type *slpA* promoter resulted in significant increases in transcription (4.3-, 4.1-, and 10.7-fold, respectively). These results indicate that the wild-type *slpA* promoter sequence was not optimal and could be improved considerably in *L. fermentum* BR11. The presence of a consensus -35 hexamer enhanced transcription (compared to the wild-type promoter) to the same level as a consensus -10 hexamer in the present study (Table 1), indicating

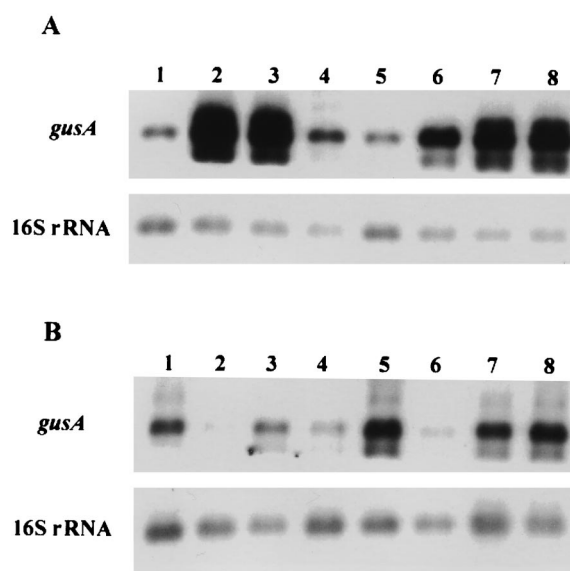


FIG. 2. Northern blot analysis of RNA isolated from *L. fermentum* BR11 (A) and *L. rhamnosus* GG (B) harboring the *slpA* wild-type and derivative promoter constructs. Membranes were hybridized with DIG-labelled *gusA* and 16S rRNA probes as described in Materials and Methods. Lanes 1, pNZslp (wild type); lanes 2, pNZTG; lanes 3, pNZT10; lanes 4, pNZT35; lanes 5, pNZT1035; lanes 6, pNZS10; lanes 7, pNZS35; lanes 8, pNZS1035. Signals were quantified by densitometry and normalized for relative plasmid copy number. The relative transcriptional activities determined are given in Table 1.

that the consensus identities at the  $-35$  and  $-10$  hexamers were of equivalent importance in the *slpA* promoter in *L. fermentum* BR11. Interestingly, transcription directed from the *slpA* promoter in *L. fermentum* BR11 was not amenable to further enhancement by the introduction of multiple consensus motifs. *gusA* transcript levels produced from the *slpA* promoter derivative harboring concurrent consensus hexamer motifs (in pNZS1035) were not significantly different from levels produced from either of the derivatives harboring individual consensus hexamers (in pNZS35 and pNZS10) in *L. fermentum* BR11 (Table 1). This observation suggests that excessive RNAP-promoter contact in the hexamer regions may have resulted in the disruption of subsequent steps of transcription initiation in *L. fermentum* BR11, sufficient to counteract the additional interaction at the hexamer motifs. Similar hypotheses have been postulated for some consensus promoters of *E. coli* (5, 7).

The TG motif significantly increased the wild-type *slpA* promoter activity in *L. fermentum* BR11 (Table 1), presumably by providing an additional RNAP contact point in the presence of the weaker (i.e., nonconsensus) hexamers (1). Furthermore, the TG motif alone conferred approximately threefold more strength than a "perfect" hexamer consensus sequence in *L. fermentum* BR11. In contrast, the TG motif displayed either no significant effect or a down-effect on transcription when introduced into a promoter which harbored consensus hexamers (cf. pNZS35 and pNZT35, pNZS10 and pNZT10, and pNZS1035 and pNZT1035) (Table 1). These data are consistent with observations in *B. subtilis*, where the TG motif is only of benefit in the context of weak  $-35$  and  $-10$  hexamers (18, 19).

The results with the modified promoter sequences in *L. rhamnosus* GG contrasted with those obtained for *L. fermentum* BR11. The introduction of individual consensus  $-35$  and  $-10$  hexamers and a TG motif all resulted in decreased transcription (3-, 13.3-, and 6.7-fold, respectively) compared to the wild-type *slpA* promoter in *L. rhamnosus* GG (Table 1). These data would suggest that the wild-type *slpA* promoter was operating near maximal strength in *L. rhamnosus* GG. Presumably, any increase in transcription initiation facilitated by the increased RNAP-promoter interaction at the consensus motifs in *L. rhamnosus* GG was exceeded by the significant disruption of later stages of transcription initiation (e.g., promoter clearance [7]).

The effect of introducing consensus hexamers on *slpA*-directed transcription in *L. rhamnosus* GG was clearly context dependent. Both the  $-35$  and  $-10$  consensus hexamers displayed up-effects on transcription when introduced into a promoter harboring a consensus hexamer at the alternate position, regardless of the status of the TG motif (Table 1). For example, introducing a consensus  $-10$  hexamer to the promoter derivative harboring a consensus  $-35$  hexamer resulted in a significant 3-fold (cf. pNZS35 and pNZS1035) or 13-fold (cf. pNZT35 and pNZT1035) increase in transcription ( $P < 0.01$ ). Similarly, the addition of a consensus  $-35$  hexamer to the promoter harboring a consensus  $-10$  hexamer led to a 13-fold increase ( $P < 0.01$ ) in relative *gusA*-specific transcript levels, regardless of the TG motif status (cf. pNZS10 and pNZS1035; cf. pNZT10 and pNZT1035). These results suggest that a consensus hexamer is able to compensate for tight binding at the alternate hexamer in *L. rhamnosus* GG. This presumably occurs by increasing the efficiency of another step in the transcription initiation process, such as providing optimal bending in the promoter spacer region or facilitating open complex formation.

Introduction of a TG motif upstream of the *slpA*  $-10$  hex-

amer in an otherwise wild-type promoter resulted in a 6.7-fold decrease in a 6.7-fold decrease in promoter activity in *L. rhamnosus* GG (Table 1). Interestingly, however, the TG motif partially compensated (2.1-fold increase [ $P < 0.01$ ]) for the down-effect of the  $-10$  consensus hexamer in this strain, regardless of the status of the  $-35$  hexamer (cf. pNZS10 and pNZT10; cf. pNZS1035 and pNZT1035). These results are in agreement with data obtained from gram-negative bacteria (6, 8) and suggest that the TG motif is able to enhance transcription in the context of a tightly bound  $-10$  hexamer in *L. rhamnosus* GG, either by alleviating strong RNAP-promoter contacts in the vicinity of the  $-10$  hexamer or by facilitating open complex formation. Interestingly, only the *slpA* promoter derivative harboring all three consensus motifs (in pNZT1035) led to a promoter activity which was significantly higher (2.1-fold) than the wild-type *slpA* promoter in *L. rhamnosus* GG. This observation is consistent with the hypotheses extended for the context-dependent changes in this strain. Comparative analysis suggested that *L. rhamnosus* GG and *L. fermentum* BR11 were capable of transcribing *gusA* mRNA to similar levels when the optimal *slpA* promoter derivative (in pNZT1035 and pNZT10, respectively) was provided (Table 1).

In the presence of a consensus  $-35$  hexamer, the TG motif displayed a significant down-effect (2.1- to 3.4-fold [ $P < 0.05$ ]) on transcription from the *slpA* promoter in both *L. rhamnosus* GG (cf. pNZS35 and pNZT35) and *L. fermentum* BR11 (cf. pNZS35 and pNZT35; cf. pNZS1035 and pNZT1035) (Table 1). Voskuil and Chambliss (18) postulated that in the presence of an efficient  $-35$  hexamer, the TG motif may act by promoting or stabilizing a transcription initiation step. The data obtained in the present study suggest that the presence of both a consensus  $-35$  hexamer and a TG motif may overstabilize transcription intermediates and thereby hinder later stages of the transcription process in *Lactobacillus* strains.

This investigation provides the first insight into the functional relevance of the  $-35$  and  $-10$  hexamers and the TG motif within promoters of *Lactobacillus*. The findings presented should facilitate the selection or development of promoter sequences for achieving high-level heterologous gene expression in these medically and industrially important organisms.

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