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The Sequence of Spacers between the Consensus Sequences Modulates the Strength of Prokaryotic Promoters

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We constructed a library of synthetic promoters for *Lactococcus lactis* in which the known consensus sequences were kept constant while the sequences of the separating spacers were randomized. The library consists of 38 promoters which differ in strength from 0.3 up to more than 2,000 relative units, the latter among the strongest promoters known for this organism. The ranking of the promoter activities was somewhat different when assayed in *Escherichia coli*, but the promoters are efficient for modulating gene expression in this bacterium as well. DNA sequencing revealed that the weaker promoters (which had activities below 5 relative units) all had changes either in the consensus sequences or in the length of the spacer between the −35 and −10 sequences. The promoters in which those features were conserved had activities from 5 to 2,050 U, which shows that by randomizing the spacers, at least a 400-fold change in activity can be obtained. Interestingly, the entire range of promoter activities is covered in small steps of activity increase, which makes these promoters very suitable for quantitative physiological studies and for fine-tuning of gene expression in industrial bioreactors and cell factories.

Metabolic engineering has promising perspectives with respect to improving the properties and performances of microorganisms used as industrial bioreactors, as cell factories, and in food fermentations. The importance of tuning gene expression in this context, i.e., to perform metabolic optimization rather than massive overexpression or gene inactivation, is now far more appreciated. However, the more subtle approach of metabolic optimization is hampered by the lack of proper expression systems for tuning gene expression in many microorganisms. Also, the fundamental understanding of a biological system through metabolic control analysis (5, 10) requires the tuning of enzyme activities in order to calculate the so-called control coefficients. For some organisms, expression systems that allow for changing gene expression for scientific purposes and for a limited set of experimental conditions have been developed. Thus, for *Escherichia coli*, the *lac* system, the *c*I-regulated lambda p_R/p_L, and many derivatives of these systems have been widely applied, and such systems have also been adapted for use in other organisms (for a recent review, see reference 12). With respect to changing steady-state gene expression, these systems can sometimes be difficult to apply, particularly when it comes to changing gene expression on an industrial scale. Besides, in most food fermentation processes, the addition of chemicals as inducers of gene expression or the changing of other process parameters is not acceptable; in such cases, there are virtually no expression systems available for tuning gene expression and thus for performing accurate metabolic optimization.

Lactic acid bacteria are widely used in food fermentation, e.g., cheese and yoghurt production, but besides lactic acid, these bacteria excrete a spectrum of organic compounds. Some of these are desirable with respect to the development of texture and flavors or for bioconservation purposes, and some are undesirable for similar or different reasons. The lactic acid bacteria are therefore obvious candidates for attempts to optimize the pattern of formation of these compounds for specific applications. But the experimental tools for manipulating gene expression are not well developed for these bacteria. An exception is the nisin-inducible system, developed recently by de Ruyter et al. (2). This system appears to be well suited for inducing gene expression in *Lactococcus lactis* by adding the antibiotic nisin (which is accepted as a food additive). A question that perhaps needs to be addressed in this context is whether the nisin expression system is also suitable for achieving a steady level of gene expression. In addition, for effective metabolic optimization, it is often necessary to optimize the expression of a number of genes, which is not feasible with the systems developed so far.

Here we describe a method for tuning steady-state gene expression in *L. lactis*. We overcome many of the limitations discussed above by using libraries of synthetic promoters which cover a wide range of promoter activities and show that the strength of prokaryotic promoters can be modulated by randomizing the spacer sequences that separates the consensus sequences. The system is food grade and well suited for use in industrial bioreactors and food fermentation processes. In addition, the system should be applicable to a broad range of biological systems. (Potential commercial users should be aware that the approach for obtaining the synthetic promoters, as well as the promoter sequences, were filed for patent worldwide [7a]).

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strain BOE270 (1) is highly competent with respect to transformation and was derived from strain MT102, which in turn is an *hsdR* derivative of strain MC1000 [*araD139* Δ(*ara-leu*)*7679 galU galK* Δ(*lac*)*174 rpsL thi-1* (1a))]. BOE270 was used for studying promoter activities in *E. coli* as well as for cloning purposes and propagation of plasmid DNA in *E. coli*. The plasmid-free *L. lactis* subsp. *cremoris* strain MG1363, which does not express β-galactosidase activity (4), was used for studying promoter activities in *L. lactis*.

The promoter cloning vector pAK80 (7) was used for cloning the synthetic promoters DNA fragments. pAK80 is a shuttle vector for *L. lactis* and *E. coli*, conferring erythromycin resistance to the host cells. The vector carries the promoterless *lacL* and *lacM* genes from *Leuconostoc lactis* (which codes for β-galactosidase enzyme activity). It contains a multiple cloning site for the insertion of DNA fragments harboring putative promoter signals, just upstream

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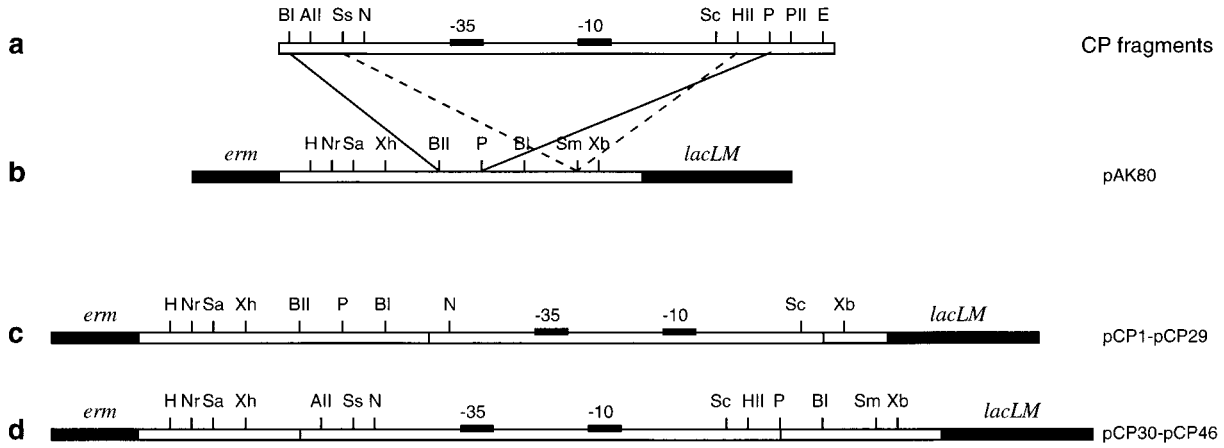

FIG. 1. Strategies used for cloning synthetic promoter fragments into the promoter cloning vector pAK80. (a) Double-stranded DNA fragments carrying putative promoter activities. (b) Restriction map and schematic representation of the relevant parts of the promoter cloning vector. The stippled and solid lines show the strategies used for cloning pCP1 through pCP29 and pCP30 through pCP46, respectively. (c) Restriction map of clones pCP1 through pCP29. (d) Restriction map of clones pCP30 through pCP46. Note that a number of clones have been subject to cloning artifacts and thus may have a slightly different restriction map. BI, *Bam*HI; AII, *Afl*II; Ss, *Ssp*I; N, *Nsi*I (*Pst*I compatible); Nr, *Nru*I; Sc, *Sca*I; HII, *Hin*cII; P, *Pst*I; PII, *Pvu*II; E, *Eco*RI; Sa, *Sac*I; Xh, *Xho*I; BII, *Bgl*II; Sm, *Sma*I; Xb, *Xba*I (not drawn to scale).

the promoterless *lacL* and *lacM* genes from *Leuconostoc lactis*. Together, the *lacL* and *lacM* genes codes for a β-galactosidase.

Enzymes. Restriction enzymes, Klenow DNA polymerase, calf intestine phosphatase, and T4 DNA ligase were obtained from and used as recommended by Pharmacia and New England Biolabs.

Oligonucleotides. Oligonucleotides were obtained from Hobolth DNA Synthesis (Hillerød, Denmark).

Second-DNA-strand synthesis. The single-stranded promoter oligonucleotides were converted to double-stranded DNA, using a 10-bp oligonucleotide (5′-CC GAATTCAG) complementary to the 3′ end of the promoter oligonucleotide as primer for the second-strand synthesis by the Klenow fragment of DNA polymerase I.

Cloning of synthetic DNA fragments into the promoter cloning vector pAK80. Two different cloning strategies were used (Fig. 1). In strategy A, the mixture of DNA fragments was digested with two restriction enzymes, *Hin*cII and *Ssp*I, and pAK80 was digested with *Sma*I. In strategy B, the mixture of DNA fragments was digested with two restriction enzymes, *Bam*HI and *Pst*I, and pAK80 was digested with *Bgl*II and *Pst*I. In both strategies, the promoter fragments were then ligated to the compatible vector fragments. The ligation mixtures were then transformed into Ca^{2+}-competent cells (13) by using a standard transformation procedure (13), and the transformation mixture were plated (at 30°C) on LB plates containing erythromycin (200 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 100 μg/ml). A total of 150 erythromycin-resistant transformants were obtained; all were white initially, but after prolonged incubation (up to 2 weeks at 4°C), a number had become blue to various extents. Later, we discovered that the development of blue color from *E. coli* colonies (but not *L. lactis* colonies) expressing *lacLM* is greatly enhanced by adding 1% glycerol to the transformation plates (data not shown). Plasmids were isolated from these blue colonies, and it was confirmed by restriction enzyme analysis that most of these clones had promoter fragments inserted in the multiple cloning site of pAK80, in the orientation that would direct transcription into the β-galactosidase gene (*lacLM*). The 46 colonies isolated had become blue to various extents; 29 from cloning strategy A (containing plasmids pCP1 through pCP29) and 17 from strategy B (containing plasmids pCP30 through pCP46) were picked for further analysis. The two weakest promoter clones, pCP31 and pCP43, did not contain a promoter fragment, and four promoter clones, pCP18, pCP19, pCP33, and pCP44, turned out to be identical to pCP27, pCP22, pCP35, and pCP45, respectively. Indeed, the activities of these sets were almost identical, which also demonstrates the reproducibility of the assay used here. The chances that two identical sequences would have arisen by coincidence during the oligonucleotide synthesis is of course negligible, and these four clones must therefore be the result of a cell division that took place after the plasmids were transformed but before the cells were plated.

Transformation of *L. lactis*. Cells of *L. lactis* subsp. *cremoris* MG1363 (4) were made competent by growth overnight in GM17 medium containing 2% glycine as described by Holo and Ness (6). Plasmid DNA from the 46 clones described above was then transformed into these cells by electroporation (6). The cells were allowed to regenerate in SGM17 medium for 2 h and then plated on SR plates containing erythromycin (2 μg/ml) and X-Gal (100 μg/ml).

β-Galactosidase assay. The assay was done as described by Miller (14) and modified by Israelsen et al. (7). Cultures carrying the plasmid derivatives of pAK80 were grown in rich medium overnight at 30°C. The medium used for *L. lactis* was M17 medium supplemented with erythromycin (2 μg/ml) and 1% glucose; for *E. coli*, LB medium supplemented with erythromycin (200 μg/ml) was used. The results presented are averages of measurements of the activities of at least three individual cultures of each clone. The standard errors were less than 30% for *E. coli* activities and less than 20% for *L. lactis* activities. Aliquots of 25 to 100 μl of the cultures were used in the β-galactosidase assay except in the case of the weakest promoter clones, where up to 2 ml of culture was concentrated and used in the assay.

RESULTS

The purpose of this work was to generate a library of synthetic constitutive promoters as a tool for genetic engineering of *L. lactis*. The promoters should cover a wide range of promoter activities, in small steps of activity changes, so that they would be applicable to quantitative physiological studies and for metabolic optimization. The following strategy was used: (i) design and synthesize a degenerated oligonucleotide sequence that encodes consensus sequences for *L. lactis* promoters, separated by spacers of random sequences; (ii) convert this mixture of oligonucleotides to double-stranded DNA fragments, using DNA polymerase and a short oligonucleotide primer complementary to the 3′ end of the degenerated oligonucleotide; and (iii) clone this mixture of DNA fragments into a promoter probing vector. The idea behind this strategy is that even though the consensus sequences should be important elements of an efficient promoter, the context in which the consensus sequences are located may modulate the strength of the promoters to some extent.

Design and construction of synthetic promoters for *L. lactis*. A considerable number of promoters have been cloned and sequenced from *L. lactis* (see the review by de Vos and Simons [3]). From these data, we extracted extended consensus sequence motifs for *L. lactis* promoters (Fig. 2A). The Pribnow box or the −10 sequence TATAAT and the −35 sequence TTGACA, known to be present in many prokaryotic promoters, are also well conserved for *L. lactis*. In addition, the sequence TG is often found 1 bp upstream of the −10 sequence; it is also possible to determine a consensus sequence for the 4 bp immediately upstream of the −35 motif, ATTC. Nilsson and Johansen (16) found well-conserved sequences among promoters of the rRNA operons: AGTTT at position −44 and GTACTGTT at positions +1 to +8. In addition to these mo-

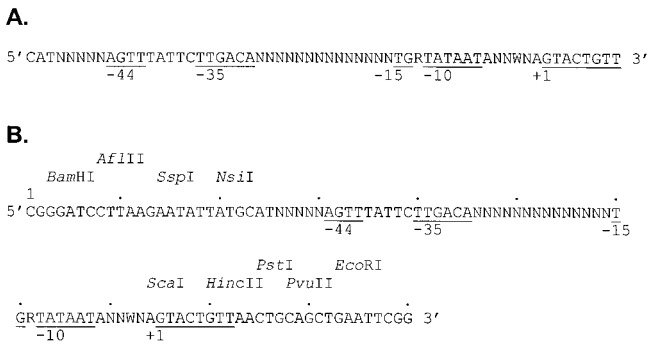

FIG. 2. Oligonucleotide sequence used for the generation of a library of synthetic promoters for *L. lactis*. (A) Consensus sequence for *L. lactis* promoters derived from data published in the literature. N = 25% each A, C, G, and T; R = 50% each A and G; W = 50% each A and T. (B) The design of the oligonucleotide. The sequence contains a number of recognition sequences for restriction endonucleases, for use in the subsequent cloning strategy. Note that the sequence from positions +1 to +8, which is a putative stringent response site, can be deleted in the cloning process if necessary. See text for further details.

tifs, two semiconserved base pairs were included, R (=A or G) upstream of the −10 sequence and W (=A or T) at position −3. Based on these data, we designed an oligonucleotide which also encodes recognition sites for multiple restriction enzymes (Fig. 2B). This mixture of oligonucleotides was converted to double-stranded DNA fragments, using a short primer complementary to the 3′ end. Finally, the resulting double-stranded DNA fragments, encoding potential promoter structures, were cloned into the polylinker on the promoter probe vector, pAK80 (7), upstream of the promoterless β-galactosidase gene, using *E. coli* as a host; this resulted in plasmids pCP1 through pCP46.

Activities of the synthetic promoters in *L. lactis*. Plasmids, pCP1 through pCP46 were then transformed into *L. lactis* subsp. *cremoris* MG1363. The different plasmids gave rise to colonies exhibiting very different intensities of blue on plates containing X-Gal. The specific activities of β-galactosidase in liquid cultures of these clones were then determined (Fig. 3) and found to vary from 0.3 Miller unit, or from slightly above the activity found with the cloning vector pAK80 without any insert, to up to more than 2,000 Miller units. Together, the promoters covered 3 to 4 logs of promoter activities in small steps of activity change.

Sequence analysis of the CP promoters. A very interesting point is the molecular basis for the differences in strength of the CP promoters, and we therefore took on the task of sequencing the promoter clones. Eighteen clones were perfect in the sense that they had the DNA sequence that was specified by the oligonucleotide (Fig. 4). The activities of these 18 promoter clones covered, in small steps of activity change, a 50-fold range of activity, from 34 up to 1,800 Miller units. Four of the CP promoters had a 16-bp spacer between the −35 and −10 sequences instead of the 17 bp specified in the oligonucleotide sequence, and the activities carried by these four clones were weak, ranging from 0.7 to 12 Miller units. Four clones had base pair changes in the −35 sequence, and two had base pair changes in the −10 sequence; those clones also had rather weak activity (0.3 to 69 Miller units).

Some clones had 1-bp deletions or a base pair change outside the −35 to −10 region or have been subject to other cloning artifacts. However, the activities of these promoter clones were all within the range covered by the perfect clones, i.e., activities from 58 to 2050 Miller units, which indicates that in this case, consensus sequences outside the −35 to −10 sequence are of little importance with respect to determining the promoter strength.

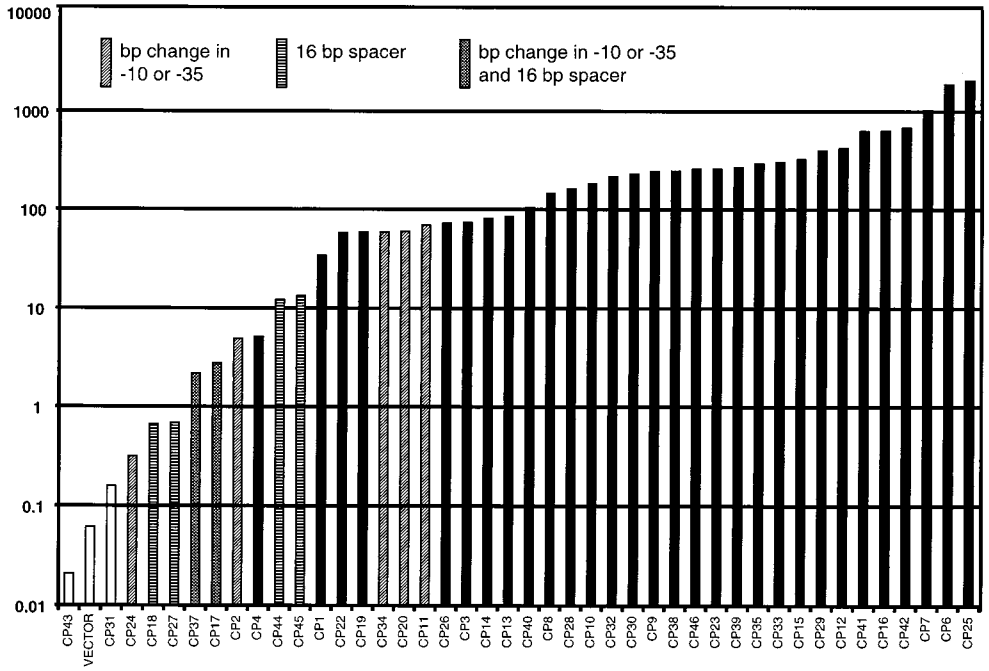

FIG. 3. Library of synthetic promoters for *L. lactis*. Promoter activities (Miller units) were assayed from the expression of a reporter gene (*lacLM*) encoding β-galactosidase transcribed from the different synthetic promoter clones on the promoter cloning vector pAK80. The patterns of the data points indicate which promoter clones contain errors in either the −35 or the −10 consensus sequence or in the length of the spacer between these sequences.

Promoter		Spec. β-gal activity	
		L. lactis	*E. coli*
Oligoseq	**CAT**NNNNN**AGTTTATTCTTGACA**NNNNNNNNNNNNNNN**TGRTATAATA**NNW**NAGTACTGTT**		
CP24	**CAT**GGGTA**AGTTTATTCTTCACA**CTATCTGGGCCCGA**TGGTATAATA**AGTG**ACTACTGTT**	0.3	3.1
CP18=CP27	**CAT**TTTGC**AGTTTATTCTTGACA**TTGTGTGCTTCGGG**TG-TATAATA**-C**TAAGTACTGTT**	0.7	0.2
CP37	**CAT**CATTA**AGTTTATTCTTCACA**TTGGCCGGAATTGT**TG-TATAATA**CC**TTAGTACTGTT**	2.2	16
CP17	**CAT**TCTGG**AGTTTATTCTTGAC-**CGCTCAGTATGCAG**TGGTATAATA**GTA**CAGTACTGTT**	2.7	10
CP2	**CAT**TTGCT**AGTTTATTCTTGACA**TGAAGCGTGCCTAA**TGGTATATTA**CT**TGAGTACTGTT**	4.9	2.8
CP4	G**AT**GTTTT**AGTTTATTCTTGACA**CCGTATCGTGCGCG**TGATATAAT**CGGGATCCTTAAGA	5.1	1.3
CP44=CP45	**CAT**CGGGT**AGTTTATTCTTGACA-**ATTAAGTAGAGCC**TGATATAATA**GT**CAGTACTGTT**	12	34
CP1	**CAT**ACCGG**AGTTTATTCTTGACA**GTTCCACCTCGGGT**TGATATAATA**TC**TCAGTACTGTT**	34	3.1
CP19=CP22	**CAT**CGCTT**AGTTT-TTCTTGACA**GGAGGGATCCGGGT**TGATATAATA**-GT**TAGTACTGTT**	58	3.3
CP34	**CAT**CGCGA**AGTTTATTCTTCACA**CACCGCAGAACTTG**TGGTATAATA**CA**ACAGTACTGTT**	59	8.4
CP20	**CAT**GGGTG**AGTTTATTCTTGACA**GTGCGGCCGGGGGC**TGATATCATA**GC**AGAGTACTATT**	60	22
CP11	**CAT**AAGTG**AGTTTATTCTTGACC**CGGACGCCCCCCTT**TGATATAATA**AGT**-AGTACTGTT**	69	1.4
CP26	**CAT**CTAC**AGTTTATTCTTGACA**TTGCACTGTCCCCC**TGGTATAATA**ACTATACATGCAT	72	10
CP3	**CAT**CCTGT**AGTTTATTCTTGACA**CAAGTCGTTAGCTG**TGGTATAATA**GG**AGAGTACTGTT**	74	0.9
CP14	**CAT**GACGG**AGTTTATTCTTGACA**CAGGTATGGACTTA**TGATATAATA**AAA**CAGTACTGTT**	81	0.3
CP13	**CAT**GCTTT**ACTTTATTCTTGACA**AAACCACCAGCTTT**TGGTATAATA**CGT**GAGAACTGTT**	84	1.0
CP40	**CAT**AGAAC**AGTTTATTCTTGACA**TTGAATAAGAAGGC**TGATATAATA**GC**-CAGTACTGTT**	104	19
CP8	**CAT**TCTTT**AGTTTATTCTTGACA**AACGTATTGAGGAC**TGATATAATA**GG**TGAGTACTGTT**	146	1.2
CP28	**CAT**GGGGCC**GTTTTATTCTTGACA**ACGGCGAGCAGACC**TGGTATAATA**AT**ATAGTACTGTT**	161	0.9
CP10	**CAT**GGCTT**AGTTTATTCTTGACA**GGGTAGTATCACTG**TGATATAATA**GG**ACAGTACTGTT**	181	2.5
CP32	**CAT**ACGGG**AGTTTATTCTTGACA**TATTGCCGGTGTGT**TGGTATAATA**ACT**TAGTACTGTT**	214	60
CP30	**CAT**GACAG**AGTTTATTCTTGACA**GTATTGGGTTACTT**TGGTATAATA**GT**TGAGTACTGTT**	228	42
CP9a	**CAT**AGTCT**AGTTTATTCTTGACA**CGCGGTCCATTGGC**TGGTATAATA**ATT**TAGTACTGTT**	243	56
CP38	**CAT**AGAGA**AGTTTATTCTTGACA**GCTAACTTGGCCTT**TGATATAATA**CA**TGAGTACTGTT**	244	92
CP46	**CAT**GATGT**AGTTTATTCTTGACA**CTGAGAGGGCCTCT**TGATATAATA**GT**TGAGTACTGTT**	256	33
CP23	**CAT**GTAGG**AGTTTATTCTTGACA**GATTAGTTAGGGGG**TGGTATAATA**TC**TCAGTACTGTT**	257	2.6
CP39	**CAT**TGCGA**AGTTTATTCTTGACA**GTACGTTTTTACCA**TGATATAATA**GT**ATAGTACTGTT**	266	0.5
CP33=CP35	**CAT**GTTGG**AGTTTATTCTTGACA**TACAATTACTGCAG**TGATATAATA**GG**TGAGTACTGTT**	298	7.0
CP15	**CAT**TACGT**AGTTTATTCTTGACA**GAATTACGATTCGC**TGGTATAATA**TA**TCAGTACTGTT**	322	1.5
CP29	**CAT**CGGTA**AG-TTATTCTTGACA**TCTCAGGGGGGACG**TGGTATAATA**AC**TGAGTACTGTT**	399	1.4
CP12b	**CAT**ATACA**AGTTTATTCTTGACA**CTAGTCGGCCAAAA**TGATATAATA**CC**TGAGTACTGTT**	419	101
CP41	**CAT**CCGCA**AGTTTATTCTTGACA**GCTGAATGTAGACG**TGGTATAATA**GT**TAAGTACTGTT**	624	18
CP16	**CAT**TGTGT**AGTTTATTCTTGACA**GCTATGAGTCAATT**TGGTATAATA**--**ACAGTACT**CAG**	627	0.3
CP42	**CAT**TCGTA**AGTTTATTCTTGACA**CCTGAGATGAGGCG**TGATATAATA**AAT**AAGTACTGTT**	680	7.2
CP7	**TAT**GCGGT**AGTTTATTCTTGACA**TGACGAGACAGGTG**TGGTATAAT**GGGTCTAGATTAGG	1021	134
CP6	**CAT**GTGGG**AGTTTATTCTTGACA**CAGATATTTCCGGA**TGATATAATA**ACT**GAGTACTGTT**	1876	280
CP25	**C-T**TTGGC**AGTTTATTCTTGACA**TGTAGTGAGGGGGC**TGGTATAAT**CACA**TAGTACTGTT**	2050	528

FIG. 4. Sequence of the area from positions −52 to +8 (relative to the putative transcription initiation site) of the synthetic promoter clones pCP1 through pCP46. The clones are ordered according to strength. Matches to the oligonucleotide consensus sequence (given at the top) are in boldface. Errors in the −35 or −10 consensus sequence and deletions in the spacer between these sequences are underlined. Two clones, CP9 and CP12, had two promoter fragments inserted in tandem, a (upstream fragment) and b (downstream fragment). In these cases, only one of the two tandem promoters was perfect; data for these promoters are shown. β-gal, β-galactosidase.

Regulation of promoter activities. The synthetic CP promoters were designed to be constitutive. To test this experimentally, the expression in exponential growth phase and stationary growth phase was measured for a selection of the promoter clones. We found that the specific activity of β-galactosidase was two- to fourfold higher in the stationary-phase cultures than in the exponential-phase cultures (data not shown). However, the copy number of the vector used in these studies has been shown to increase approximately threefold in the stationary phase (11), which demonstrates that the CP promoters are indeed quite close to being constitutive under these conditions.

Activities of the synthetic promoters in *E. coli*. Another interesting point is whether the promoters are functional in other organisms, and if so, whether the relative strength of the promoters would be dependent on the organism. As described above, the promoter cloning vector, pAK80, that we used here for construction of the synthetic promoters also replicates in *E. coli*; indeed, the promoter clones were first isolated in *E. coli*. We could therefore measure the activities of the synthetic promoters also in *E. coli* (Fig. 5). The promoter strength was also highly variable for the individual promoters in this organism, and we found that the promoters covered activities from 0.2 to 500 Miller units. In this case also, the activity increased in small steps.

The absolute values of β-galactosidase units measured in *E. coli* were lower on average compared to *L. lactis*; this was probably a consequence of a low efficiency of translation of the *lacL* and *lacM* genes in *E. coli*, since these genes and their ribosome binding sites originate from the gram-positive bacterium *Leuconostoc mesenteroides*. When some of the strongest promoters were cloned into a promoter cloning vector designed for *E. coli*, the promoters turned out to be quite strong (data not shown).

Figure 6 shows a plot of activity of the CP promoters in *L. lactis* and *E. coli*. The strengths of the individual CP promoters in the two organisms correlate somewhat but not very well: some promoters which were quite strong in *L. lactis* were relatively weak in *E. coli*, and vice versa. Moreover, the pattern that we observed in *L. lactis*, i.e., that the relatively strong promoters were the perfect ones, did not hold true for *E. coli*: here the promoters which had either an error in the consensus sequence or a shorter spacer were relatively strong.

DISCUSSION

We have constructed a library of synthetic promoters that differ in strength over 3 to 4 logs of activity, and this range of activity is covered by small steps of activity increase. Moreover, some of the promoters that resulted from this random approach turned out to be quite strong.

The fact that the library of promoters covered such a wide range of activities was somewhat surprising to us; the underlying idea behind the construction of the CP promoters was that the context of the consensus sequences (the spacers) would play a role in modulating the strength of a promoter, rather than changing the activity over several logs of activity. Indeed, much of that variation (below 5 Miller units) was probably a consequence of the accidental introduction of mutations in the consensus sequences and in the length of the spacer regions. In contrast, the strong promoters in *L. lactis* (those having activities higher than 100 Miller units) were all perfect with respect to the consensus sequence and spacer length. But even when we confine our analysis to these promoter clones, we find 400-fold variation in promoter activity, still in small steps of activity increase, which demonstrates that the context in which the consensus sequences are embedded (i.e., the spacers) clearly is important for promoter strength.

The ranking of the promoters depended on the organism in

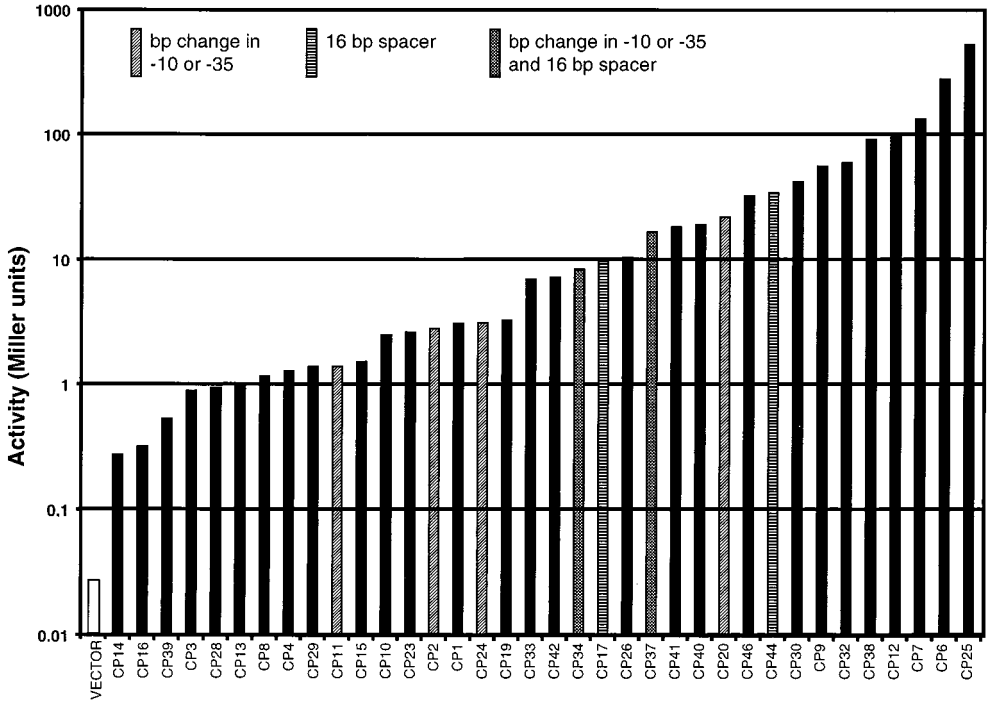

FIG. 5. β-Galactosidase activities of the CP promoters in *E. coli*. The promoter activities were assayed from the expression of a reporter gene (*lacLM*) encoding β-galactosidase transcribed from the different synthetic promoter clones on the promoter cloning vector pAK80. The patterns of the data points indicate which promoter clones contained errors in either the −35 or the −10 consensus sequence or in the length of the spacer between these sequences. See text for further details.

which they were measured, possibly because the σ factor-RNA polymerase complexes that recognize these promoters have different structures in the two organisms due to differences in amino acid sequences. The fact that *E. coli* accepted some of the less perfect CP promoters as relatively strong promoters could indicate that *E. coli* is more promiscuous with respect to promoter structure than *L. lactis*. This makes some sense considering the composition of the *L. lactis* genome: the AT content is 65%, which is much closer to the base composition of the −35 and −10 consensus sequences. These sequences are therefore more likely to accidentally occur in *L. lactis*, and a stricter requirement for promoter sequences might therefore be expected for this organism.

The process of transcription initiation consists of several events (reviewed in reference 17). First, recognition and binding of the σ factor-RNA polymerase complex to the promoter region takes place (closed complex formation). Subsequently, there is local melting of the DNA double helix (open complex formation), possibly assisted by local negative DNA supercoiling. Finally, the binding between the σ factor-RNA polymerase complex and the promoter area must dissociate and clear the promoter area, so that another initiation complex may form. From this model, it is clear that efficient binding between the σ factor-RNA polymerase complex and the promoter area does not guarantee a strong promoter; promoter strength must be a compromise between binding, melting, and clearance, and probably other factors as well.

What then controls the strength of the individual synthetic promoters presented here? It does not appear that any additional conserved sequence motifs have been generated among the strongest promoters. Rather, it seems that the overall three-dimensional structure which arises from a particular nucleotide sequence could be important.

The method presented here for tuning gene expression in the living cell has both advantages and disadvantages compared to the methods that would use an inducible expression system such as the *lac* promoter. A disadvantage is that instead of only one genetic construct, perhaps three to four constructs have to be made. On the other hand, the constructs are made

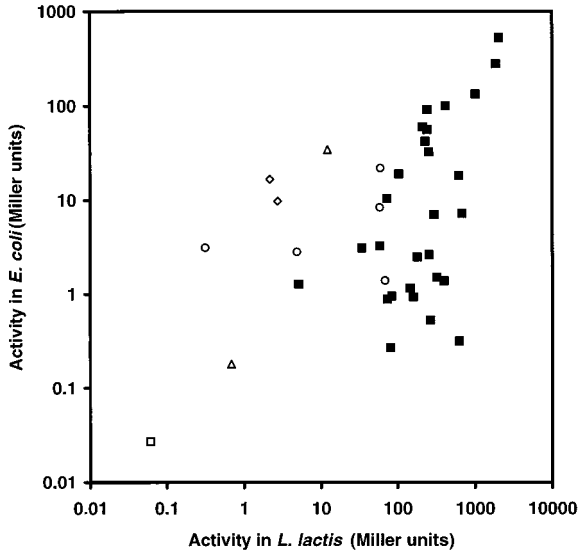

FIG. 6. Correlation between promoter activities in *L. lactis* and *E. coli*. The promoter activities measured in *E. coli* (from Fig. 5) were plotted as a function of the promoter activities measured in *L. lactis* (from Fig. 3). The symbols indicate errors in either the −35 or −10 sequence (solid circles), a 16-bp spacer (triangles), or promoters with both of these errors (diamonds). The open square represents the vector clone.

in parallel, so that the amount of work should not be proportional to the number of constructs. The inducible systems have the advantage that gene expression can be turned on at the proper time during a fermentation, which is sometimes essential (for instance, when the product is toxic to the host cell). The work presented here was aimed at generating a library of constitutive promoters, for achieving a constant level of gene expression throughout the growth of a culture. We are currently working on synthetic inducible promoters in which a regulatory motif has been added. This should allow us to generate libraries of promoters, which differ in basal expression level and can be induced to various extents, by changing a fermentation parameter (i.e., temperature, pH, or salt concentration) or by adding a specific inducer.

The system presented here also has advantages. One is that it is easier to attain a steady expression level of the enzyme in question, which is often quite difficult with inducible systems such as the *lac* system (8). With the method presented here, once the optimal expression level of the enzyme has been determined, the engineered strain is ready to use directly in the fermentation process.

An important feature of the system described here, in a longer perspective, is the possibility to simultaneously modulate, to different extents, the expression of several individual genes or operons located at various positions of the genome in the same strain. Metabolic control analysis (5, 10) showed that in theory, flux and concentration control can be shared among several enzymes in a pathway, and experimental determinations of flux control have often showed that control seems to be distributed over many enzymes in the living cell (9, 15, 18, 19, 22, 23): in most cases, there may not be such a thing as a rate-limiting step, and even if one finds a step that has a measurable control, the control will often disappear relatively quickly as the enzyme is being overexpressed. Since the sum of flux control must equal unity, this then means that flux control has been shifted to other steps in the pathway. In summary, in order to increase a given flux in a living cell, it may thus be necessary to (i) optimize the individual expression of several genes and (ii) after one round of optimization in which one enzyme was clamped at the optimal level, continue the optimization of other enzymes in the pathway. With the systems available until now, one would then quickly run out of expression systems to use, but with our method, one can in principle continue the optimization numerous times.

In this report, the method for generating synthetic promoters of different strengths was illustrated for use in the gram-positive bacterium *L. lactis*. However, there is no obvious reason why the approach should be limited to this organism, and the fact that the same promoter library was also functional in the gram-negative bacterium *E. coli* suggests that the approach may be universally applicable to prokaryotic organisms. An exciting question is then, can the approach be extended to work for modulating gene expression in eukaryotic cells? Such experiments are under way, and the results are quite encouraging.

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