Promoters from *Corynebacterium glutamicum*: cloning, molecular analysis and search for a consensus motif

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Relatively limited information about promoter structures in *Corynebacterium glutamicum* has been available until now. With the aim of isolating and characterizing such transcription initiation signals, random *Sau3A* fragments of *C. glutamicum* chromosomal DNA and of the corynebacterial phage ϕ GA1 were cloned into the promoter probe vector pEKplCm and selected for promoter activity by chloramphenicol resistance of transformed *C. glutamicum* cells. The nucleotide sequence of ten chromosomal and three phage fragments was determined and the transcriptional start (TS) sites were localized by primer extension analyses. Additionally, the promoters of five previously isolated *C. glutamicum* genes were cloned and mapped. All of the isolated promoters were also functional in the heterologous host *Escherichia coli*. A comparative analysis of the newly characterized promoter sequences together with published promoters from *C. glutamicum* revealed conserved sequences centred about 35 bp (ttGcca) and 10 bp (TA.aaT) upstream of the TS site. The position of these motifs and the motifs themselves are comparable to the –35 and –10 promoter consensus sequences of other Gram-positive and Gram-negative bacteria, indicating that they represent transcription initiation signals in *C. glutamicum*. However, the *C. glutamicum* consensus hexamer of the –35 region is much less conserved than in *E. coli*, *Bacillus*, *Lactobacillus* and *Streptococcus*.

Keywords: *Corynebacterium glutamicum*, promoter, consensus sequence

INTRODUCTION

The basic DNA sequence pattern of bacterial promoters recognized by the major RNA polymerase σ -factors (e.g. σ^{70} of *Escherichia coli* or σ^A of *Bacillus subtilis*) seems to be common to many bacterial species (Hawley & McClure, 1983; Lisser & Margalit, 1993; Pouwels & Leer, 1993; Haldenwang, 1995; Helmann, 1995). The first proposal for a promoter consensus sequence was based on align-

ment and statistical evaluation of 46 *E. coli* promoter sequences (Rosenberg & Court, 1979). This consensus sequence which is partially conserved in all σ^{70} and σ^A promoters comprises the two hexamers TTGaca and TAtaaT, separated by 17 ± 1 bp and located about 35 bp and 10 bp, respectively, upstream of the transcriptional start (TS) site. The importance of these conserved –35 and –10 regions for the function of *E. coli* promoters was further confirmed by mutational analysis and by alignment of larger sets of promoters (e.g. Hawley & McClure, 1983; Lisser & Margalit, 1993; Harley & Reynolds, 1987). For Gram-positive bacteria, a consensus sequence based on 29 vegetative promoters, mainly from the genera *Bacillus* and *Staphylococcus*, was proposed by Graves & Rabinowitz (1986). In addition to –35 and –10 consensus sequences, which were identical to those found for *E. coli* σ^{70} promoters, the authors detected a conserved TG motif located 1 bp upstream of the –10 hexamer and, with a higher than 50% conservation rate, an A-track at

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Abbreviations: CAT, chloramphenicol acetyltransferase; Cm, chloramphenicol; Km, kanamycin; MCS, multiple cloning site; TS, transcriptional start; wt, wild-type.

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position -43 relative to the TS site. The significance of these features was very recently corroborated by compilation and analysis of 236 *B. subtilis* σ^A -dependent promoter sequences (Helmann, 1995). TG dinucleotides at position -15/-14 and A+T-rich regions upstream of the -35 region were also observed in several promoters from *E. coli* (Galas *et al.*, 1985; Deuschle *et al.*, 1986; Belyaeva *et al.*, 1993) and from some other Gram-positive bacteria, e.g. *Lactobacillus* and *Streptococcus* (Pouwels & Leer, 1993; van der Vossen *et al.*, 1987; Morrison & Jaurin, 1990; Matern *et al.*, 1994).

Corynebacterium glutamicum is a Gram-positive, non-sporulating micro-organism known particularly as a producer of various amino acids. Although a variety of *C. glutamicum* genes have been characterized (reviewed in Jetten & Sinskey, 1995), knowledge on gene expression signals is still poor and the general structure of *C. glutamicum* promoters, i.e. a typical consensus sequence, has not as yet been defined. So far, the TS sites, and thus the promoter regions, of only a few *C. glutamicum* genes have been identified experimentally (Peoples *et al.*, 1988; von der Osten *et al.*, 1989; Marcel *et al.*, 1990; Han *et al.*, 1990; Börmann *et al.*, 1992; Follettie *et al.*, 1993; Heery & Dunican, 1993; Schwinde *et al.*, 1993; Eikmanns *et al.*, 1994). Some of these promoter regions display in their -10 region a 6 bp motif similar to the typical -10 consensus sequence of σ^{70} and σ^A promoters. However, only one of the promoters, i.e. that of the *fda* gene (von der Osten *et al.*, 1989), shows similarity to both the -10 and -35 consensus sequences and a proper distance of 18 bp between them. According to the few data available, it seemed that promoter sequences in *C. glutamicum* differ from those of *E. coli* or *B. subtilis* or at least display less conformity with the typical promoter consensus pattern common to both Gram-negative and Gram-positive bacteria.

In this study we isolated 18 promoters from *C. glutamicum* and four from the temperate coryneophage ϕ GA1 (Sonnen *et al.*, 1990) and mapped the respective TS sites. Then, using newly developed computer programs, we performed a comparative analysis of these promoter sequences together with the previously published ones to find a common pattern representing a corynebacterial promoter consensus sequence.

METHODS

Bacterial strains, plasmids and growth conditions. *C. glutamicum* wild-type (wt) (ATCC 13032), the restriction-deficient *C. glutamicum* R163 (Brabetz *et al.*, 1991) and *E. coli* DH5 (Hanahan, 1985) were used. For isolation of promoters, the promoter probe vector pEKplCm (Eikmanns *et al.*, 1991) was employed. This vector carries the kanamycin (Km) resistance gene and, downstream of the multiple cloning site (MCS), the promoterless *cat* gene with transcription terminators of the *E. coli rrnB* operon. The region between the MCS and the reporter gene contains translational stop codons in all three reading frames followed by the native ribosomal binding site of the *cat* gene. Further plasmids used were pJC20 containing the *dapA* and *dapB* genes (Cremer *et al.*, 1990), pBM1 containing the *ilvA* gene (Möckel *et al.*, 1992), pKK5-5 containing the *ilvC*

gene (Keilhauer *et al.*, 1993) and pKK7 containing the gene for a putative membrane protein (ORFMP) from *C. glutamicum* (Pátek *et al.*, 1994). LB medium and 2 $\times$ TY medium (Sambrook *et al.*, 1989) were used for cultivation of *E. coli* and *C. glutamicum*, respectively. When appropriate, Km (50 μ g ml⁻¹) was added to selective media. *E. coli* was grown aerobically at 37 °C, *C. glutamicum* at 30 °C.

DNA isolation and manipulation. Chromosomal DNA from *C. glutamicum* was isolated as described previously (Eikmanns *et al.*, 1994). DNA from phage ϕ GA1 (Sonnen *et al.*, 1990) was kindly provided by H. Sonnen (TU Darmstadt). Plasmids from *E. coli* were isolated as described by Birnboim (1983), those from *C. glutamicum* by the same method with prior incubation (1 h, 37 °C) of the cells with lysozyme (15 mg ml⁻¹). *E. coli* was transformed by the CaCl₂ method (Sambrook *et al.*, 1989), *C. glutamicum* by electroporation (Liebl *et al.*, 1989).

Restriction enzymes, T4 DNA ligase, Klenow polymerase, proteinase K, DNase I, RNase A, RNasin and avian myeloblastosis virus (AMV) reverse transcriptase were obtained from Boehringer Mannheim or from Promega and used as instructed by the manufacturer.

DNA sequencing of the promoter fragments was done by the dideoxy chain termination method using the Sequenase 2.0 kit from USB Amersham Life Sciences. Primers were synthesized according to sequences of plasmid pEKplCm adjacent to the MCS with the Gene Assembler Plus and chemicals from Pharmacia. Primer CmS (GGTGGTATATCCAGTGAT) covers positions +30 to +13 and primer CmR (CCGGACGCGCTCCATG) positions -117 to -100 relative to the translational start of the *cat* gene.

RNA isolation and primer extension analysis. Total RNA from *C. glutamicum* was isolated as described previously (Eikmanns *et al.*, 1994). For primer extension analyses of the promoter fragments cloned in pEKplCm, the following primers were used: CmS (see above), Cm2 (TTGGCTGCAGGTCCG-ACGG) covering positions -62 to -79, Cm3 (CGGCGGA-TTTGTCCTACT) covering positions +39 to +56 and Cm4 (GAAAATCTCGTCTGAAGCTCG) covering positions +21 to +40 relative to the translational start of the *cat* gene in plasmid pEKplCm. For determination of the TS sites from the *C. glutamicum* genes (*dapB*, *dapA*, *ilvA*, *ilvC* and *orfMP*) oligonucleotides complementary to the beginning of the individual genes were used as primers: O1-*dapB* (TCCGAGA-ACGCCAACCTTG), O2-*dapB* (CACGGCCTTTGGCTC-CG), O3-*dapB* (GCTGCCACAATAGTTTGAC), O1-*dapA* (TCCGTGAATGGAGTAACC), O2-*dapA* (CCGGTCTTA-GCTGTAAAC), O1-*ilvA* (CACTCCTGGACTTTTCTC), O2-*ilvA* (AGCTCCGCTCCGCTAGCC), O1-*ilvC* (CAGTTC-AATAGCCATGGG), O2-*ilvC* (GCCCCCACTGTAAATC-GTG), O1-*orfMP* (GGGGTGGCGAGCGCGATC) and O2-*orfMP* (GCCCCATGCGATTGTAA). All TS sites were determined using at least two primers.

Primer extension reactions were carried out as described by Eikmanns *et al.* (1994) using about 70 μ g total RNA from *C. glutamicum*, 20 pmol primer, [α -³⁵S]dATP and 5 U AMV reverse transcriptase. The primer extension products and, for exact localization of the TS site, sequencing reactions performed with the same oligonucleotides used for the primer extensions were loaded and separated on a 6% (w/v) polyacrylamide sequencing gel.

Enzyme assay and determination of MIC. To determine the chloramphenicol acetyltransferase (CAT) activity, *C. glutamicum* cells were grown in 60 ml medium to exponential growth phase, washed twice in TS buffer (50 mM Tris, 50 mM NaCl, pH 7)

and resuspended in 2 ml TS buffer. Cells were disrupted by sonication as described by Börmann *et al.* (1992) and after centrifugation for 30 min at 4 °C and 13000 *g*, the supernatant was used as cell extract. The protein concentration was determined by the biuret method (Gornall *et al.*, 1949). CAT activity was assayed photometrically at 412 nm and 37 °C as described by Shaw (1975) in 1 ml of a solution of 100 mM Tris/HCl buffer (pH 7.8), 1 mM 5,5'-dithiobis-2-nitrobenzoic acid, 0.1 mM acetyl CoA, and 0.25 mM chloramphenicol (Cm). One unit of activity was defined as 1 µmol of Cm acetylated min⁻¹.

The MIC of Cm on *C. glutamicum* and *E. coli* was determined on LB agar plates as described by Ozaki *et al.* (1984).

Computer analysis. In general two methods have been used to search for a consensus pattern in a set of promoter sequences. In the first method the sequences are aligned according to the TS site or according to an arbitrarily chosen region (e.g. the -10 region) and the most frequent bases in each column are counted (Hawley & McClure, 1983; Graves & Rabinowitz, 1986). The other method is based on the occurrence of a sequence motif, i.e. a *k*-letter pattern word, in a distinct region of sequences defined by the distance from the TS site (Galas *et al.*, 1985). Since the sequence motifs sought may not occur in a completely conserved form, nor at a precise distance from the TS, two further parameters, *W* and *m*, must be set: *W* (window width) is the number of admitted consecutive columns to be examined at a time and *m* is the minimal number of matches taken into account.

In our analysis we employed both these methods and combined their results. At first, all promoter sequences were aligned from base -50 to +10 with respect to the experimentally determined TS site. No prior assumptions about the significance of any sequence pattern or motif were made. In the procedure corresponding to the first method we developed and used the PROBAB program which, for a given number of sequences (*q*) examines the number of base occurrences *n_x* (where *x* = A, C, G or T) in each column *i* (*i* = 1-60) of the alignment. As a measure of orderliness of a particular column the program calculates the probability of base distribution for each column in a set of given sequences (*P*):

$$P = \frac{q!}{n_A! \cdot n_C! \cdot n_G! \cdot n_T!} \times \frac{1}{4^q}$$

Local minimums of *P* within the alignment indicate regions in which the sequences under consideration are more correlated (similar) than in other parts of the alignment.

For the other approach to sequence analysis, we developed the PROMSCAN program. The strategy of this program is very similar to that described by Galas *et al.* (1985) and it identifies conserved sequence motifs: from all possible combinations of *k* bases, the algorithm localizes in each of the individual sequences a *k*-letter word (a sequence of *k* bases) for which the number of matches within the alignment of all sequences is maximal within a given window. The sum of the maximal numbers of matches over all the sequences is then considered as a measure of conservation of the *k*-letter word examined. The *k*-letter word with the highest number of matches is considered the *k*-letter pattern word. The measure of significance of the *k*-letter pattern words is given by the relative score *S_{rel}* which is the sum of matches in a given window related to its maximal value (at *k* = 6 and *q* = 33 the maximal value is 6 × 33 = 198). The PROBAB and PROMSCAN programs are available from M. Pátek.

RESULTS

Cloning of promoter-carrying fragments from *C. glutamicum* and from phage φGA1

The promoter probe vector pEKplCm was used for the cloning and identification of promoter-active DNA fragments from the chromosome of *C. glutamicum* and from the corynebacterial phage φGA1. *C. glutamicum* cells harboring pEKplCm were sensitive to Cm at > 2 µg ml⁻¹, indicating that no transcription from vector-borne promoters occurred.

Complete *Sau3A* digests of chromosomal DNA from wt *C. glutamicum* and of phage φGA1 were ligated into the *Bam*HI site of pEKplCm. The ligation mixtures were transformed into *E. coli* DH5 and transformants were selected on medium containing Km. About 10000 transformants of the ligation mixture containing *C. glutamicum* DNA and about 2000 of that containing φGA1 DNA were pooled. Plasmids from both pools were isolated, transformed into *C. glutamicum* R163 and the Km-resistant transformants obtained were screened for Cm resistance by replica-plating onto medium containing Cm at 5 µg ml⁻¹. Approximately 1 % of the transformants containing pEKplCm with *C. glutamicum* DNA and 5 % of those containing pEKplCm with φGA1 DNA grew on this medium, indicating that the fragments cloned in these *C. glutamicum* isolates contain functional promoters directing the transcription of the *cat* gene in pEKplCm. Restriction analysis of plasmids from 20 Cm-resistant clones carrying pEKplCm with *C. glutamicum* DNA revealed insert sizes ranging from 70 bp to 600 bp. Plasmid analysis of 20 clones carrying pEKplCm with φGA1 DNA revealed three types of inserts with sizes of about 100 bp, 200 bp and 250 bp, suggesting that we obtained three promoter-carrying fragments from phage φGA1.

Besides shotgun-cloning of promoter-active fragments from the wt *C. glutamicum* chromosome and from phage φGA1, DNA fragments carrying the upstream regions of five previously sequenced *C. glutamicum* genes, *dapA*, *dapB*, *ilvA*, *ilvC* and *orfMP*, were cloned. For this purpose, the 0.7 kb *Pst*I fragment (F-dapA) from plasmid pJC20, the 0.8 kb *Bcl*I-*Sal*I fragment (F-dapB) from plasmid pJC20, the 0.8 kb *Hind*III-*Bam*HI fragment (F-ilvA) from plasmid pBM1, the 0.8 kb *Sal*I fragment (F-ilvC) from plasmid pKK5-5 and a 0.3 kb *Sau3A* fragment (F-orfMP) from plasmid pKK7 were ligated in the correct orientation into the MCS of pEKplCm and the resulting plasmids were introduced into *C. glutamicum* R163. All *C. glutamicum* transformants grew on medium containing Cm at 30 µg ml⁻¹, indicating that the fragments contained the promoters responsible for transcription of the respective genes.

The 10 smallest promoter fragments obtained by shotgun cloning from the *C. glutamicum* chromosome (designated F1, F2, F10, F13, F22, F34, F37, F45, F64 and F75), the three promoter fragments obtained from phage φGA1 (PF101, PF104 and PF109) and the five promoter fragments from the *C. glutamicum* genes (F-dapA, F-dapB, F-

Table 1. Size and promoter activity of DNA fragments isolated from the chromosome of *C. glutamicum* and from phage ϕ GA1

Promoter activity was assayed by determining the MIC of Cm and by measuring the specific activity of CAT in cell extracts of *C. glutamicum* harbouring the respective pEKplCm derivative.

Fragment in pEKplCm	Fragment size (bp)	MIC of Cm in <i>C. glutamicum</i> ($\mu\text{g ml}^{-1}$)	MIC of Cm in <i>E. coli</i> ($\mu\text{g ml}^{-1}$)	CAT activity in <i>C. glutamicum</i> [U (mg protein) $^{-1}$]*
None		< 2	40	< 0.001
F13	81	35	360	0.04 $\pm$ 0.01
F22	115	45	240	0.07 $\pm$ 0.01
F2	157	45	240	0.10 $\pm$ 0.02
F75	72	50	200	0.11 $\pm$ 0.01
F-orfMP	314	45	400	0.12 $\pm$ 0.01
F34	266	50	280	0.15 $\pm$ 0.02
F1	129	65	400	0.16 $\pm$ 0.02
F10	147	50	500	0.17 $\pm$ 0.01
PF109	206	45	450	0.19 $\pm$ 0.01
F-dapA	709	40	200	0.20 $\pm$ 0.02
F64	145	55	200	0.23 $\pm$ 0.02
F37	102	55	600	0.25 $\pm$ 0.02
PF104	94	60	600	0.41 $\pm$ 0.04
PF101	252	55	600	0.42 $\pm$ 0.07
F-ilvA	~ 800	50	360	0.92 $\pm$ 0.05
F-ilvC	809	55	450	1.12 $\pm$ 0.10
F45	182	60	600	1.16 $\pm$ 0.14
F-dapB	813	55	560	1.70 $\pm$ 0.15

* The values are means $\pm$ SD obtained from at least three independent experiments.

ilvA, F-ilvC and F-orfMP) were chosen for a more detailed analysis.

Assessment of promoter activity in *C. glutamicum* and *E. coli*

The relative strength of the cloned promoters was assessed by determining the MIC of Cm and by measuring the specific CAT activities in the different *C. glutamicum* clones. Whereas *C. glutamicum* harbouring plasmid pEKplCm was inhibited by 2 $\mu\text{g Cm ml}^{-1}$ and showed no detectable CAT activity, all clones harbouring pEKplCm with promoter fragments displayed Cm resistance to > 30 $\mu\text{g ml}^{-1}$ and CAT activities of 0.04 to 1.7 U (mg protein) $^{-1}$ (Table 1). As also observed for *E. coli* and *B. subtilis* (Goldfarb *et al.*, 1981; Close & Rodriguez, 1982), the relationship between resistance level and CAT activity was not linear, although the reason for this discrepancy remains unclear. We previously showed that the copy numbers of pEKplCm and promoter-carrying derivatives thereof are very similar (Eikmanns *et al.*, 1991) and thus, major errors in evaluation of promoter strength due to different reporter gene dose can be ruled out.

To test whether the cloned *C. glutamicum* promoters function in *E. coli*, the MICs of Cm were also determined

for *E. coli* DH5 harboring pEKplCm or its derivatives. As shown in Table 1, all plasmids containing promoter fragments conferred much higher Cm resistance to *E. coli* than plasmid pEKplCm. Although higher by a factor of 5–10, the relative MIC values obtained for the *E. coli* clones roughly correlated to those observed in *C. glutamicum* clones carrying the respective plasmids. Fragments F37, PF104, PF101, F45 and F-dapB conferred relatively high Cm resistance to both *C. glutamicum* and *E. coli*, whereas fragments F13, F22, F2 and F-dapA led to relatively low levels of Cm resistance in both bacteria. The most conspicuous exception to this correlation is the promoter on fragment F64 which was relatively strong in *C. glutamicum* but one of the weakest in *E. coli*. However, the results indicated that all fragments isolated from the *C. glutamicum* chromosome and from phage ϕ GA1 contain promoters which were functional in both *C. glutamicum* and *E. coli*.

Sequencing of promoter-carrying fragments and mapping of TS sites

The complete nucleotide sequences of the 13 shotgun-cloned promoter-carrying fragments were determined from both strands. The sizes of the fragments are given in Table 1 and promoter-relevant parts of the sequences, as

		TS (+1)
		↓
P1-1	TCAACTAAGCGTCCC <u>ATAAGA</u> ACGAGGCGCAAGGCTGATG <u>TACT</u> CTGTCA	A CCATGGATA
P2-1	TGTACTCTGTCAACCATGGA <u>TAA</u> ACCGGTCTGAGGGATG <u>CAGC</u> GTCTGCT	G ATTTTTCGC
P-2	GTAATTGGGGCGCGTGGAGCCATTTCGGCTTCAGTAGT <u>ACTTT</u> ATTACT	A GCTGCTGTG
P-10	AGACCAAGGATGGCTTTGAAGTTCTTGAGATCCGCAGTACGGTATTAA	A GAAAATGAT
P-13	GATCCGTCCCGGGGAAGTGGGAAATGCT <u>AAAA</u> TGGAACG	A CATTGCGAT
P1-22	CATTTTCTAATTCGTGTGTAGCTTTGAATTGGCCTTGGTGAATCCAGGC	T TATGGTTAT
P2-22	GTGTGTAGCTTTGAATTGGCCCTTGGTGAATCCAGGCTTATGGTTATCTCT	G CAGCTATT
P-34	GAAACGTGGCGACTTATGGGATTGAATGCAACGTGATGGGGTAGCGGAC	C CCAACCAAA
P-37	TCCTAATAAATATTTGGGAGGGTTCGCGGGATTAATGTACTCTCGAAGGTT	G AACACAGGG
P-45	TTGGTCAGGGATTTTTCGCCGAGGCACTAATTTTGCTAAAGTAAGTGAC	G AAGAAGTTC
P-64	AGATTCTGCGCGAGTTCCGCCACACGTCATCATTACCTATGATGAGAAC	G GCGGTTACC
P-75	TAGCGATTAGCGCGCGCTGAGCTTTAGTTTACAGCTAACATGAGGTGCAT	A AACAAAACG
P1-101	ACAATCGAACCAGCAGACCCGACGCCCTCGATAACCTGGGTTGTGCGGT	T TTGTTATTC
P2-101	AACCTGGGTTGTGCGGTTTGTATTCTCTTGAGTGAGTTCGTGGGGTCTC	C ATTTGGGTT
P-104	ATGAGCTGGCAGCGTATTTGACCGATCCGCACACCTGGGATAATGTGTGG	A TTTTGTCCG
P-109	GTGGAAGCCCATGAAGAGCACATCATGAAACTGTGGAGGTATTGACCGGT	G GATGAATTC
P-ORFMP	TGACAGGTGCTACTTCGCCGAGCAACTCTTTAGTCAACTACCCGTAATCAA	G TGCAAAGCA
P- <i>ilvA</i>	ACCTATGCCAAAGTAGGTGCAATTCTAGGAGAAGATTACACTAGTCAACC	A TGAGTGAAA
P- <i>ilvC</i>	TGTGACTAGTCAAACACCGCTCTAATTACATGTGTGTGGTAGAACAATAAT	G TAGTTGTCT
P- <i>dapA</i>	TGCGGGGTTGTTTAAACCCCAAAATGAGGGAAGAAGGTAACTTGAATCT	A TGAGCACAG
P1- <i>dapB</i>	TAGGTATGGATATCAGCACCTTCTGAACGGGTACGCTCAGACTGGTGGGC	G TTTGAAAAA
P2- <i>dapB</i>	CGTTTGAAAACTCTTCGCCCCACGAAAAATGAAGGAGCATAATGGGAATC	A AGGTTGGCG
P- <i>hom</i>	AATTTGCGGGCTTTAAAGCAAAAATGAACAGCTTGGTCTATAGTGGCTAG	G TACCCTTTT
P- <i>thrC</i>	CATATTTGAGACGGGTGTTGGGGAGTATTGTGTACCCCTTGGGATAGGGT	T ATATCCGTG
P- <i>fda</i>	AAGGAAATATCACACGACAAAAGTTGAGTGATGCAGGCATAATTGGCTAT	A GGCAACTGA
P- <i>lysA</i>	TAAGTATGGGTCTGATTCTGTGCGACGGGTGTACCTCGGCTAGAATTCT	C CCCATGACA
P1- <i>ask</i>	AAAACACTCTCTGCTAGGTAGACACAGTTTATAAAGGTAGAGTTGAGC	G GGTAACGT
P2- <i>ask</i>	TGAATACGCTCGTGCATTCATGTTGCCACTTCGCGTACGCTCGTCTTATA	G TAATGATCC
P- <i>gdh</i>	TTTTAATTCCTTTGGTCTATATCTGTGCGACACTGCCATAATTGAAAGT	G AGCAGTTAC
P- <i>glt</i>	TCTCACTTCGGATATGGCTAAACCGCATTATCGGTATAGCGTGTAAAC	G GACCAGATT
P- <i>gap</i>	ATGATTTTGATCTGCTGCGAAATCTTTGTTTCCCGCTAAAGTTGAGGA	C AGGTTGACA
P- <i>pgk</i>	CAATTGAATACCGGTTGCCAGCGCCACACAATGTGTGGCAATCTGGGACA	G TGCATCACA
P- <i>trp</i>	GGAACTACGCAAGAACCCCAAAATGATTAATAATTGAGACAAGCTTCCC	A CTTATGTGA

Fig. 1. Nucleotide sequences of *C. glutamicum* promoters aligned according to their TS sites. Putative -35 and -10 regions identified by the PROMSCAN program are underlined. The promoter sequences of *hom*, *thrC*, *fda*, *lysA*, *ask*, *gdh*, *glt*, *gap*, *pgk* and *trp* were taken from references cited in the text.

deduced from primer extension analyses (see below), are shown in Fig. 1.

To map the precise TS sites within the shotgun-cloned promoter fragments, total RNA was isolated from *C. glutamicum* clones containing the promoter-carrying plasmids and used for reverse transcription with the CmS, Cm2, Cm3 and/or Cm4 primers. For mapping the TS sites of *dapA*, *dapB*, *ilvA*, *ilvC* and *orfMP*, total RNA from wt *C. glutamicum* and gene-specific primers were used. In all cases at least one band was detected as primer extension product. As examples, the results of the experiments with fragments F2 and PF109, and of those with the genes *orfMP* and *ilvC* are shown in Fig. 2. The nucleotide sequences of all promoters from nucleotide -50 to +10 with respect to the TS site were designated P-1, P-2, P-13 and so on (according to F1, F2, F13 and so on) and are aligned in Fig. 1. In the cases of F1, F22, PF101 and *dapB* the primer extension patterns indicated two distinct TS

sites and thus the presence of two promoters (designated P1-1 and P2-1, P1-22 and P2-22, P1-101 and P2-101, and P1-*dapB* and P2-*dapB*, respectively). The distances between the two TS sites were 38, 14, 33 and 49 bp in the cases of F1, F22, PF101 and *dapB*, respectively. For 14 promoters we observed two or more adjacent or nearby bands apparently representing multiple TS sites from one promoter, e.g. in the cases of PF109 and F-*ilvC* (Fig. 2). The presence of multiple TS sites was previously observed for several promoters of *E. coli* and *Streptomyces* genes (Hawley & McClure, 1983; Strohl, 1992) and, thus, is not uncommon.

In 26 cases out of 33, the first transcribed base was either A or G. Such a preference for a purine residue as the TS site has been reported for *E. coli* and lactic acid bacteria (Hawley & McClure, 1983; van der Vossen *et al.*, 1987).

All promoter sequences (from position -50 to +1)

Fig. 2. Primer extension analyses of fragments F2 (a), PF109 (b), F-orfMP (c) and F-ilvC (d). The primer extension products are shown in lanes 1, 2, 3 and 4, respectively. Lanes A, C, G and T represent the sequencing reactions using the same primers as for the primer extensions. The relevant DNA sequences are shown on the right. Note that the sequences represent the coding strands and are thus complementary to those shown in Fig. 1.

display a relatively high A + T content of up to 67%. In some promoters, e.g. P-*orfMP*, P-64, P-*gdb* and P-*lysA*, the region between positions -35 and -85 is extremely A + T-rich (65 to 78%). Aside from the high A + T content, a frequent occurrence of the dinucleotide TG (169 compared to the statistical average of 121) is the most apparent feature of the nucleotide composition of the whole set of promoter sequences.

Analysis of promoter sequences

To find conserved regions, the promoter sequences were aligned according to the TS site and analysed by our programs *PROBAB* and *PROMSCAN*. In those cases where two or more adjacent or nearby primer extension signals were found, we always took the strongest signal as the TS site. The analysis was done with the set of 22 promoters described here together with 11 published promoter sequences, i.e. P-*hom* (Peoples *et al.*, 1988), P-*thrC* (Han *et al.*, 1990), P-*fda* (von der Osten *et al.*, 1989), P-*lysA*

(Marcel *et al.*, 1990), P1-*ask* and P2-*ask* (Follettie *et al.*, 1993), P-*gdb* (Börmann *et al.*, 1992), P-*glt* (Eikmanns *et al.*, 1994), P-*gap* and P-*pgk* (Schwinde *et al.*, 1993), and P-*trp* (Heery & Dunican, 1993) which are also aligned according to the TS site in Fig. 1. At first, the probabilities of the base distribution were calculated for all positions in the set by the *PROBAB* program. In the resulting histogram (Fig. 3a) the strongest signals (i.e. the lowest probability) were seen in the region of the TS site (+1 and -1) and at positions -7 and -21, slightly weaker signals at positions -32, -34 and -43. The high value of $-\log(P)$ at position +1 corresponds to the preference for A and G nucleotides as the TS site (see above). In the next step, *PROMSCAN* was run on the same alignment of the sequences with various values for length of the k -letter word (k), window width (W) and lowest number of matches (m). For various sets of parameters there was always a clear signal in the region from position -6 to -14. For the parameters $k = 6$, $W = 11$ and $m = 4$, a conspicuous region of k -letter pattern words with high S_{rel} values was

Fig. 3. Probability of base distribution P calculated by PROBAB for each position in the promoter sequences aligned according to the experimentally determined TS sites (a), to the -10 region (b) and to the -35 region (c) as found by the PROMSCAN program. For convenience, the negative logarithm of P [$-\log(P)$] is shown.

detected 8–14 bp upstream of the TS site (Fig. 4a). This region is referred to in the following as the -10 region. When the search for a hexamer ($k = 6$) was run at different W and m values ($W = 9$ – 13 ; $m = 3$ – 5) the k -letter pattern words with the highest S_{rel} values were also in the -10

region, the single S_{rel} values being somewhat higher or lower. The hexamers with high S_{rel} values were GGTAAG, GGTAAC, TACAAT, TATAAT and TAGAGT and they were located at positions -12 to -9 relative to the TS site.

Fig. 4. Relative scores (S_{rel}) of the k -letter pattern words for each position of the window in the promoter set aligned according to the experimentally determined TS sites (a), to the -10 region (b) and to the -35 region (c) as found by the PROMSCAN program. Parameters: $k=6$; $W=11$ in (a) and 9 in (b) and (c); $m=4$.

The results obtained by the alignment according to the TS site prompted us to align the promoter sequences according to the TACAAT or the most similar motif within the -10 region. With this new alignment the probability of the base distribution was again calculated by PROBAB (Fig. 3b). Compared to the original alignment according to the TS site (Fig. 3a), several extraordinarily strengthened signals were obtained in the -10 region.

Using the same alignment and the PROMSCAN program with various W and m values, the signal peak in the -10 region was also stronger (e.g. Fig. 4b). Moreover, a second region of k -letter pattern words with above average S_{rel} values was detected about 20 bp upstream of the -10 region. This region is referred to in the following as the -35 region. The hexamers in the maximum of the -10 region were GGTATA, GGTACA, TATAAT and

Fig. 5. Base distribution in *C. glutamicum* promoters aligned according to the -10 and -35 regions found by PROMSCAN. The break was introduced at the least correlated position. The number of occurrences of the most prevalent base at each position is shown in the histogram. The number of occurrences of each base at each position is shown at the bottom. The bases which occur in at least 42% of the promoters (i.e. 14 out of 33) at the same position are listed above the histogram. Bases occurring in more than 70% of the promoters (i.e. 23 out of 33) at the same position are capitalized.

TACAAT, those in the maximum of the -35 region were TCTTGG, TTTTGC, TTTGCC, TTGCCA, GGCCAA and GCCAAA. Essentially the same results were obtained for W values of 8–11 and for m values of 3–5.

In the next step, the promoter sequences were aligned according to the sequence TTTTGCCAAA or the most similar motif in the -35 region and both the PROBAB and PROMSCAN analyses were done again. As expected, the plot of P showed strongly enlarged signals in the -35 region. The PROMSCAN analysis with W values between 8 and 11 and m values between 3 and 5 (e.g. Fig. 4c) showed identical or similar k -letter pattern words with relatively high S_{rel} values in the -35 and -10 regions as in the previous analysis.

By comparing the location of the major signals from the PROBAB and PROMSCAN analyses, it can be seen that they are correlated both in their position and strength. To define a consensus sequence and the level of conservation of single bases within this consensus sequence, the widely used base distribution analysis (Hawley & McClure, 1983) was performed. For this purpose, we aligned the promoter sequences according to the -10 and -35 motifs found by the PROMSCAN program (and underlined in Fig. 1) with a break at the least correlated position. As shown in Fig. 5, the most conserved sequences were ttTGcc.a in the -35 region and ggTA.a aT in the -10 region. It can also

be seen from Fig. 5 that the level of conservation of individual bases in the -35 and -10 consensus sequences is quite different. However, we conclude that these two motifs characterize the regions carrying the main informational content of *C. glutamicum* promoters apparent from the sequences themselves.

DISCUSSION

The aim of our study was the isolation and molecular characterization of *C. glutamicum* promoters. Using the promoter probe vector pEKpLcm, a variety of promoter-active DNA fragments could be isolated. All 18 fragments tested also drove transcription of *cat* in *E. coli*, indicating that the isolated promoters were active in this organism too. This result was not unexpected since many of the genes cloned from *C. glutamicum* were expressed in *E. coli* as indicated by heterologous complementation of appropriate auxotrophs (reviewed in Jetten & Sinskey, 1985). Conversely, several *E. coli* genes were efficiently expressed in *C. glutamicum* (e.g. Eikmanns *et al.*, 1991; Pátek *et al.*, 1989) and the *E. coli* *tac*, *lacUV5*, and *trp* promoters have been shown to be functional in corynebacteria (Morinaga *et al.*, 1987). All these results indicate that the general structure of promoters from *C. glutamicum* is similar to that of promoters from *E. coli*. However, there were also reports on corynebacteria-specific

promoters which were obviously not functional in *E. coli* (Cadenas *et al.*, 1991).

The similarity of the primary structure of promoters from *C. glutamicum* with that of promoters from other bacteria was substantiated by comparative computer analysis. Both programs applied in this study showed that in our set of *C. glutamicum* promoters the most correlated region is located around 10 bp upstream of the TS site and comprises the hexamer TA.AAT. This or a similar motif can be found in all promoters. Another conserved region was detected about 35 bp upstream of the TS site. The consensus hexamer TTGCCA found in this region differs from the *E. coli* consensus TTGACA only in the fourth position. The role of the TTGCCA motif is not that obvious since it is clearly seen (> 3 bp matching) in only 14 promoters out of the 33. In several others the motif was not easily discernible, indicating a lower conservation in *C. glutamicum* promoters. However, from the position relative to the TS site, from the spacing (a mean of 17.35 bp) and from the sequence of the two conserved hexamer motifs it is obvious that these signals in *C. glutamicum* promoters are comparable to the -10 and -35 promoter consensus sequences of *E. coli* (Hawley & McClure, 1983; Lissner & Margalit, 1993; Harley & Reynolds, 1987) and other eubacteria, e.g. *Bacillus* (Helmman, 1995; Graves & Rabinowitz, 1986; Moran *et al.*, 1982), *Lactobacillus* (Pouwels & Leer, 1993) and *Streptococcus* (van der Vossen *et al.*, 1987; Morrison & Jaurin, 1990). The significance of the *C. glutamicum* consensus motifs found by our analyses is corroborated by the fact that the *tac* promoter (11 out of 12 positions identical to consensus) was found to be very efficient in *C. glutamicum* (Eikmanns *et al.*, 1991; Morinaga *et al.*, 1987) and that an alteration of the -10 hexamer in the *E. coli lac* promoter from TATGTT to TATATT, thus increasing the similarity to the consensus sequence, led to higher efficiency of the promoter in *C. glutamicum* (Brabetz *et al.*, 1991).

Aside from the -10 and -35 hexamers, other positions seem to be conserved in *C. glutamicum* promoters: a double G next to the 5' end of the -10 element, an A residue 2 bp downstream of and a T residue directly upstream of the -35 element (Fig. 5). In fact, the conserved bases near the -10 and -35 hexamers expand the consensus motifs to ggTA.aaT and tttGcca.a, respectively. An extended promoter consensus has been proposed for Gram-positive bacteria due to the presence of a TG motif found in the majority of *Bacillus* and *Lactobacillus* promoters upstream of or separated by 1 bp from the -10 hexamer (Helmman, 1995; Graves & Rabinowitz, 1986; van der Vossen *et al.*, 1987; Matern *et al.*, 1994). Eleven out of the 33 promoters analysed here contain a TG motif in the 5' neighbourhood of the -10 hexamer and thus, the TG doublet next to the -10 hexamer might have significance for promoter function in *C. glutamicum* too. The relatively high A+T content upstream of the -10 region also seems significant especially since the mean A+T content of *C. glutamicum* is only about 43.5% (Liebl, 1991). Many promoters from Gram-positive bacteria, as well as some strong promoters

from *E. coli*, show an A+T-rich region upstream of the -35 region (e.g. Helmman, 1995; Graves & Rabinowitz, 1986; Moran *et al.*, 1982) and it has been suggested that A+T-rich sequences, in particular A tracks, are responsible for intrinsic DNA curvatures upstream of the -35 region and thus for the activation of transcription (reviewed in Perez-Martin *et al.*, 1994). Recently, Ross *et al.* (1993) showed that an A+T-rich segment at the -40 to -60 region of the *E. coli rrnB* P1 promoter, the so-called UP element, stimulates transcription from several promoters and these authors suggest that such elements function as a third recognition element for RNA polymerase in at least some bacterial promoters. However, whether the GG in front of the -10 hexamer and/or the relatively high A+T content upstream of the -10 region is important for promoter activity in *C. glutamicum* remains to be studied.

In some Gram-positive bacteria, e.g. *Bacillus* and *Streptococcus*, most of the -10 and -35 promoter regions match their consensus sequences TATAAT and TTGACA, respectively, nearly perfectly (Helmman, 1995; Graves & Rabinowitz, 1986; Morrison & Jaurin, 1990; Moran *et al.*, 1982). In contrast, this does not hold for the set of *C. glutamicum* promoters studied here. Several of the bases in the *C. glutamicum* promoter consensus motifs are only moderately conserved (see Fig. 5), indicating only relatively moderate conservation of the motif as a whole. Since the conservation level of promoter consensus sequences probably reflects the requirements of the major RNA polymerase σ -factor in a given organism (Moran *et al.*, 1982), it was interesting to compare the degree of consensus conservation per position in our study with that in compilations of promoters from other bacteria. As shown in Table 2, nearly all bases in the -35 and -10 consensus hexamers in the *Bacillus*, *Lactobacillus* and *Streptococcus* promoters are much more and those in the *E. coli* promoters are considerably more conserved than in the set of *C. glutamicum* promoters. These data suggest that the recognition specificity of the major RNA polymerase in the organisms listed decreases in the order *Bacillus*/*Lactobacillus*/*Streptococcus* > *E. coli* > *C. glutamicum*. It should be mentioned that the mean A+T content of chromosomal DNA in these (groups of) organisms decreases in the same order (Balows *et al.*, 1991). Thus, our data corroborate a presumption mentioned by Morrison & Jaurin (1990), i.e. that recognition specificity of RNA polymerase(s) and the conformity of promoters with the A+T-rich consensus sequence might have evolved in a given organism by adaptation to the A+T content of its chromosome. In further accordance with this presumption, a compilation of promoters from streptomycetes (mean A+T content of 30%) revealed that their sequences are widely diverse and that only 29 out of 139 promoters show motifs similar to the TATAAT and TTGACA hexamers in their -10 and -35 regions (Strohl, 1992).

As deduced from CAT activity measurements in *C. glutamicum*, the promoters isolated in this study varied considerably in their strength. Since the activity of promoters in *E. coli* can be correlated to a major extent

Table 2. Degree of consensus conservation in the –35 and –10 motifs in compilations of promoters from different organisms

Promoter compilations for the different organisms were taken from the following references: *C. glutamicum*, 33 promoters (this work); *E. coli*, 263 promoters, (Harley & Reynolds, 1987); *Bacillus subtilis*, 237 promoters (Helmann, 1995); *Lactobacillus*, 30 promoters (Pouwels & Leer, 1993); *Streptococcus*, 17 promoters (Morrison & Jaurin, 1990).

Bacterium	Percentage conservation of single nucleotides in the consensus motif of the:											
	–35 region						–10 region					
	T	T	G	A*	C	A	T	A	T	A	A	T
<i>C. glutamicum</i>	48	48	73	54	45	52	79	73	33	58	42	85
<i>E. coli</i>	78	82	68	58	52	54	82	89	52	59	49	89
<i>Bacillus subtilis</i>	87	83	78	64	51	58	94	96	60	78	74	94
<i>Lactobacillus</i>	90	87	60	63	43	60	83	83	60	87	63	90
<i>Streptococcus</i>	88	76	88	59	59	47	76	100	65	71	65	100

*In the *C. glutamicum* –35 consensus sequence the fourth nucleotide is C instead of A. An A at this position was found in only 7 of the 33 promoters (21 % conservation).

with their similarity to the –35 and –10 consensus hexamers (Hawley & McClure, 1983), we assumed that the observed CAT activity roughly correlates with the identity score of a given promoter with the predicted consensus promoter sequence. However, such a correlation could not be recognized. This might be due to several reasons. (i) The different 5'-untranslated mRNA sequences may have differential effects on *cat*-mRNA translatability or stability. (ii) The measured CAT activity arose in some cases from two promoters present on the fragments. (iii) Sequences outside of the consensus hexamers (e.g. A + T-rich upstream regions) can modulate promoter activity significantly (e.g. Galas *et al.*, 1985; Deuschle *et al.*, 1986; Ross *et al.*, 1993). (iv) Our set of promoter sequences may include regulated promoters. Many *E. coli* promoters under positive regulation show only random similarity to the –35 consensus motif (Raibaud & Schwartz, 1984). The presence of such promoters in our alignment would weaken the correlation between activity level and similarity to the consensus. (v) Our set of promoters may include some which should be classified separately due to their recognition by alternative RNA polymerase σ -factors of *C. glutamicum*. So far, the presence of multiple σ -factors has not been reported for *C. glutamicum*, however, the presence of more than one σ -factor has been observed in many prokaryotic genera (Lonetto *et al.*, 1992; Merrick, 1993). Aside from the predominant 'housekeeping' σ^{70} , *E. coli* possesses at least six further σ -factors (Helmann, 1991; Gross *et al.*, 1992) and in *Streptomyces coelicolor* and *B. subtilis* there are at least six and nine, respectively, additional σ -factors each having a specific target promoter sequence (Haldenwang, 1995; Buttner, 1989). If distinct σ -factors are also present in *C. glutamicum*, different sequence motifs for optimal promoter recognition and transcription initiation can be expected. Thus, the identification and characterization of the corynebacterial housekeeping σ -factor and, if present,

of additional σ -factors and the corresponding consensus sequences would lead to a more detailed picture of corynebacterial promoters.

It becomes clear from the data presented here that further work, e.g. stringent mutational and biochemical analysis of selected promoters, is necessary to gain detailed information on the structure-function relationship of promoters (or promoter classes) in *C. glutamicum* and thus a better understanding of gene expression in this industrially important organism.

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REFERENCES

- Balows, A., Trüper, H. G., Dworkin, M., Harder, W. & Schleifer, K.-H. (1991). *The Prokaryotes*. New York: Springer-Verlag.
- Belyaeva, T., Griffiths, L., Minchin, S., Cole, J. & Busby, S. (1993). The *Escherichia coli* *cysG* promoter belongs to the 'extended –10' class of bacterial promoters. *Biochem J* **296**, 851–857.
- Birnboim, H. C. (1983). A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**, 243–255.
- Börmann, E. R., Eikmanns, B. J. & Sahm, H. (1992). Molecular analysis of the *Corynebacterium glutamicum* *gdb* gene encoding glutamate dehydrogenase. *Mol Microbiol* **6**, 317–326.
- Brabetz, W., Liebl, W. & Schleifer, K.-H. (1991). Studies on the utilization of lactose by *Corynebacterium glutamicum* bearing the lactose operon of *Escherichia coli*. *Arch Microbiol* **155**, 607–612.

- Buttner, M. J. (1989). RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **3**, 1653–1659.
- Cadenas, R. S., Martín, J. F. & Gil, J. A. (1991). Construction and characterization of promoter-probe vectors for *Corynebacteria* using the kanamycin-resistance reporter gene. *Gene* **98**, 117–121.
- Close, T. J. & Rodriguez, R. L. (1982). Construction and characterization of the chloramphenicol-resistance gene cartridge: a new approach to the transcriptional mapping of extrachromosomal elements. *Gene* **20**, 305–316.
- Cremer, J., Eggeling, L. & Sahm, H. (1990). Cloning the *dapA dapB* cluster of the lysine-secreting bacterium *Corynebacterium glutamicum*. *Mol Gen Genet* **220**, 478–480.
- Deuschle, U., Kammerer, W., Gentz, R. & Bujard, H. (1986). Promoters of *Escherichia coli*: a hierarchy of *in vivo* strength indicates alternate structures. *EMBO J* **5**, 2987–2994.
- Eikmanns, B. J., Kleinertz, E., Liebl, W. & Sahm, H. (1991). A family of *Corynebacterium glutamicum*–*Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing. *Gene* **102**, 93–98.
- Eikmanns, B. J., Thum-Schmitz, N., Eggeling, L., Lüdtke, K. U. & Sahm, H. (1994). Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum* *gltA* gene encoding citrate synthase. *Microbiology* **140**, 1817–1828.
- Follettie, M. T., Peoples, O., Agoropoulous, C. & Sinskey, A. J. (1993). Gene structure and expression of the *Corynebacterium flavum* N13 *ask*–*asd* operon. *J Bacteriol* **175**, 4096–4103.
- Galas, D. J., Eggert, M. & Waterman, M. S. (1985). Rigorous pattern-recognition methods for DNA sequences. *J Mol Biol* **186**, 117–128.
- Goldfarb, D. S., Doi, R. H. & Rodriguez, R. L. (1981). Expression of Tn9-derived chloramphenicol resistance in *Bacillus subtilis*. *Nature* **293**, 309–311.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J Biol Chem* **177**, 751–766.
- Graves, M. C. & Rabinowitz, J. C. (1986). *In vivo* and *in vitro* transcription of the *Clostridium pasteurianum* ferredoxin gene. *J Biol Chem* **261**, 11409–11415.
- Gross, C. A., Lonetto, M. & Losick, R. (1992). Bacterial sigma factors. In *Transcriptional Regulation*, Vol. 1, pp. 129–176. Edited by S. McKnight & K. Yamamoto. Cold Spring Harbour, NY: Cold Spring Harbor Laboratory.
- Haldenwang, W. G. (1995). The sigma factors of *Bacillus subtilis*. *Microbiol Rev* **59**, 1–30.
- Han, K.-S., Archer, J. A. C. & Sinskey, A. J. (1990). The molecular structure of the *Corynebacterium glutamicum* threonine synthase gene. *Mol Microbiol* **4**, 1693–1702.
- Hanahan, D. (1985). Techniques for transformation of *E. coli*. In *DNA Cloning. A Practical Approach*, Vol. 1, pp. 109–135. Edited by D. M. Glover. Oxford: IRL Press.
- Harley, C. B. & Reynolds, R. P. (1987). Analysis of *E. coli* promoter sequences. *Nucleic Acids Res* **15**, 2343–2361.
- Hawley, D. K. & McClure, W. R. (1983). Compilation and analysis of *Escherichia coli* promoter sequences. *Nucleic Acids Res* **11**, 2237–3355.
- Heery, D. M. & Dunican, L. K. (1993). Cloning of the *trp* gene cluster from a tryptophan-hyperproducing strain of *Corynebacterium glutamicum*: identification of a mutation in the *trp* leader sequence. *Appl Environ Microbiol* **59**, 791–799.
- Helmann, J. D. (1991). Alternative sigma factors and the regulation of flagellar gene expression. *Mol Microbiol* **5**, 2875–2882.
- Helmann, J. D. (1995). Compilation and analysis of *Bacillus subtilis* σ^A -dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res* **23**, 2351–2360.
- Jetten, M. S. M. & Sinskey, A. J. (1995). Recent advances in the physiology and genetics of amino acid-producing bacteria. *Crit Rev Biotechnol* **15**, 73–103.
- Keilhauer, C., Eggeling, L. & Sahm, H. (1993). Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J Bacteriol* **175**, 5595–5603.
- Liebl, W. (1991). The genus *Corynebacterium* – nonmedical. In *The Prokaryotes*, Vol. 2, pp. 1157–1171. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Liebl, W., Bayerl, A., Schein, B., Stillner, U. & Schleifer, K.-H. (1989). High efficiency electroporation of intact *Corynebacterium glutamicum* cells. *FEMS Microbiol Lett* **65**, 299–304.
- Lisser, S. & Margalit, H. (1993). Compilation of *E. coli* mRNA promoter sequences. *Nucleic Acids Res* **21**, 1507–1516.
- Lonetto, M., Gribskov, M. & Gross, C. A. (1992). The σ^{70} family: sequence conservation and evolutionary relationships. *J Bacteriol* **174**, 3843–3849.
- Marcel, T., Archer, J. A. C., Mengin-Lecreulx, D. & Sinskey, A. J. (1990). Nucleotide sequence and organization of the upstream region of the *Corynebacterium glutamicum* *lysA* gene. *Mol Microbiol* **4**, 1819–1830.
- Matern, H. T., Klein, J. R., Henrich, B. & Plapp, R. (1994). Determination and comparison of *Lactobacillus delbrueckii* subsp. *lactis* DSM7290 promoter sequences. *FEMS Microbiol Lett* **122**, 121–128.
- Merrick, M. J. (1993). In a class of its own – the RNA polymerase sigma factor σ^{54} (σ^N). *Mol Microbiol* **10**, 903–909.
- Möckel, B., Eggeling, L. & Sahm, H. (1992). Functional and structural analyses of threonine dehydratase from *Corynebacterium glutamicum*. *J Bacteriol* **174**, 8065–8072.
- Moran, C. P. Jr, Lang, N., LeGrice, S. F. J., Lee, G., Stephens, M., Sonenshein, A. L., Pero, J. & Losick, R. (1982). Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. *Mol Gen Genet* **186**, 339–346.
- Morinaga, Y., Tsuchiya, M., Miwa, K. & Sano, K. (1987). Expression of *Escherichia coli* promoters in *Brevibacterium lactofermentum* using the shuttle vector pEB003. *J Biotechnol* **5**, 305–312.
- Morrison, D. A. & Jaurin, B. (1990). *Streptococcus pneumoniae* possesses canonical *Escherichia coli* (sigma 70) promoters. *Mol Microbiol* **4**, 1143–1152.
- von der Osten, C. H., Barbas, C. F., Wong, C. H. & Sinskey, A. J. (1989). Molecular cloning, nucleotide sequence and structural analysis of the *Corynebacterium glutamicum* *fda* gene: structural comparison of *C. glutamicum* fructose-1,6-biphosphate aldolase to class I and class II aldolases. *Mol Microbiol* **3**, 1625–1637.
- Ozaki, A., Katsumata, R., Oka, T. & Furuya, A. (1984). Functional expression of the genes of *Escherichia coli* in Gram-positive *Corynebacterium glutamicum*. *Mol Gen Genet* **196**, 175–178.
- Pátek, M., Krumbach, K., Eggeling, L. & Sahm, H. (1994). Leucine synthesis in *Corynebacterium glutamicum*: enzyme activities, structure of *leuA*, and effect of *leuA* inactivation on lysine synthesis. *Appl Environ Microbiol* **60**, 133–140.
- Pátek, M., Navrátil, O., Hochmannová, J., Nesvera, J. & Hubáček, J. (1989). Expression of the threonine operon from *Escherichia coli* in

- Brevibacterium flavum* and *Corynebacterium glutamicum*. *Biotechnol Lett* **11**, 231–236.
- Peoples, O. P., Liebl, W., Bodis, M., Maeng, P. J., Follettie, M. T., Archer, J. A. & Sinskey, A. J. (1988). Nucleotide sequence and fine structural analysis of the *Corynebacterium glutamicum* *hom-thrB* operon. *Mol Microbiol* **2**, 63–72.
- Perez-Martin, J., Rojo, F. & de Lorenzo, V. (1994). Promoters responsive to DNA bending: a common theme in procaryotic gene expression. *Microbiol Rev* **58**, 268–290.
- Pouwels, P. H. & Leer, R. J. (1993). Genetics of lactobacilli: plasmids and gene expression. *Antonie van Leeuwenhoek* **64**, 85–107.
- Raibaud, O. & Schwartz, M. (1984). Positive control of transcription initiation in bacteria. *Annu Rev Genet* **18**, 173–206.
- Rosenberg, M. & Court, D. (1979). Regulatory sequences involved in the promotion and termination of RNA transcription. *Annu Rev Genet* **13**, 319–353.
- Ross, W., Gosink, K. K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K. & Gourse, R. L. (1993). A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. *Science* **262**, 1407–1413.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning, a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schwinde, J. W., Thum-Schmitz, N., Eikmanns, B. J. & Sahm, H. (1993). Transcriptional analysis of the *gap-pgk-tpi-ppc* gene cluster of *Corynebacterium glutamicum*. *J Bacteriol* **175**, 3905–3908.
- Shaw, W. V. (1975). Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Methods Enzymol* **43**, 737–755.
- Sonnen, H., Schneider, J. & Kutzner, H. J. (1990). Characterization of ϕ GA1, an inducible phage particle from *Brevibacterium flavum*. *J Gen Microbiol* **136**, 567–571.
- Strohl, W. R. (1992). Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res* **20**, 961–974.
- van der Vossen, J. M., van der Lelie, D. & Venema, R. (1987). Isolation and characterization of *Streptococcus cremoris* Wg2-specific promoters. *Appl Environ Microbiol* **53**, 2452–2457.
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