Mol Gen Genet (1983) 190:384–389

Expression of Antibiotic Resistance Genes from *Escherichia coli* in *Bacillus subtilis*

Jürgen Kreft, Klaus J. Burger, and Werner Goebel
Institut für Genetik und Mikrobiologie der Universität Würzburg, Röntgenring 11, D-8700 Würzburg, Federal Republic of Germany

Summary. Bifunctional recombinant plasmids were constructed, comprised of the *E. coli* vectors pBR322, pBR325 and pACYC184 and different plasmids from Gram-positive bacteria, e.g. pBSU161-1 of *B. subtilis* and pUB110 and pC221 of *S. aureus*. The beta-lactamase (*bla*) gene and the chloramphenicol acetyltransferase (*cat*) gene from the *E. coli* plasmids were not transcribed and therefore not expressed in *B. subtilis*. However, tetracycline resistance from the *E. coli* plasmids was expressed in *B. subtilis*. Transcription of the tetracycline resistance gene(s) started in *B. subtilis* at or near the original *E. coli* promoter, the sequence of which is almost identical with the sequence recognized by σ^{55} of *B. subtilis* RNA polymerase.

Introduction

The Gram-positive soil bacterium *Bacillus subtilis* is considered harmless for man and animals and is widely used in basic and applied microbiology. Efforts have been made to apply the techniques of molecular cloning, which are highly developed for the Gram-negative enterobacterium *Escherichia coli*, to *B. subtilis* (Ehrlich 1978; Goebel et al. 1979; Lovett and Keggins 1979; Dubnau et al. 1980) and several plasmid vector systems have been reported. These involve plasmids capable of replication in *B. subtilis* alone or in *B. subtilis* and other Gram-positive microorganisms (Ehrlich 1977; Gryczan et al. 1978; Wilson and Baldwin 1978; Bernhard et al. 1978; Gryczan and Dubnau 1978; Ehrlich et al. 1982) or alternatively bifunctional plasmids able to replicate in both *E. coli* and *B. subtilis* (Ehrlich 1978; Kreft et al. 1978; Kreft and Hughes 1982). In this paper we describe the construction of bifunctional plasmids which allow studies on the expression in *B. subtilis* of three antibiotic resistance genes from *E. coli*.

Materials and Methods

Bacterial Strains. E. coli 5K, r^-, m^- was obtained from S. Glover, and JC1569 *recA*, *thy*$^-$, *arg*$^-$, *his*$^-$, *met*$^-$, *leu*$^-$, Sm^r harbouring pACYC184 from S. Cohen. *B. subtilis* BR151CM1 *trpC2, metB10, lys3, spoCM1* was kindly provided by P. Lovett.

Offprint requests to: J. Kreft

Source of Reagents. All reagents if not otherwise stated were obtained from Merck, FRG. The antibiotics used were a gift from Bayer, FRG. Sodium dodecyl sulfate (SDS), ethidium bromide, polyethylene glycol type 6000, lysozyme and pancreatic RNase were purchased from Serva, FRG.

Enzymes. The restriction enzymes *Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I, *Pst*I, *Xba*I and T4 DNA ligase were obtained from BRL (USA) or Biolabs (USA). S1 nuclease was purchased from BRL.

Growth of Strains, Cell Lysis and Purification of Plasmid DNA were performed as previously described (Mayer et al. 1977; Bernhard et al. 1978).

Cleavage of DNA and Electrophoresis in Agarose Gels. Digestion with restriction enzymes were carried out as indicated by the manufacturers. The reactions were stopped by heating the reaction mixture to 68° C for 8 min or by addition of EDTA (100 mM final conc.). Electrophoresis was carried out in 1% agarose slab gels in 36 mM Tris-HCl, 30 mM NaH_2PO_4, 10 mM EDTA, pH 7.5 (Meyers et al. 1975).

Construction of Hybrid Plasmids and Transformation. The in vitro construction of hybrid plasmids (Cohen et al. 1973) using T4 DNA ligase, transformation of *E. coli* and of *B. subtilis* protoplasts have been described (Goebel and Bonewald 1975; Kreft et al. 1978; Chang and Cohen 1979).

Purification of RNA. Isolation of RNA from *B. subtilis* BR151 was performed according to Glisin et al. (1974) by cell lysis with sarkosyl (4% w/v) and subsequent centrifugation through a cushion of CsCl ($\rho = 1.71$ g/cm) in 0.1 M EDTA pH 8.0. The RNA from the pellet was treated with DNAseI (Worthington) in the presence of ribonuclease inhibitor (vanadyl-ribonucleoside complex, BRL) to remove traces of contaminating DNA.

DNA/RNA Hybridization. The dot-blot method was used according to Kafatos et al. (1979) using 25–100 µg of RNA per dot and gene specific DNA probes labeled by nick-translation ($0.5–1.0 \times 10^6$ cpm/µg) with α-^{32}P-dNTPs according to de la Cruz et al. (1980).

S1 Nuclease Mapping. Transcripts were analyzed by the method of Berk and Sharp (1977). The experiments were

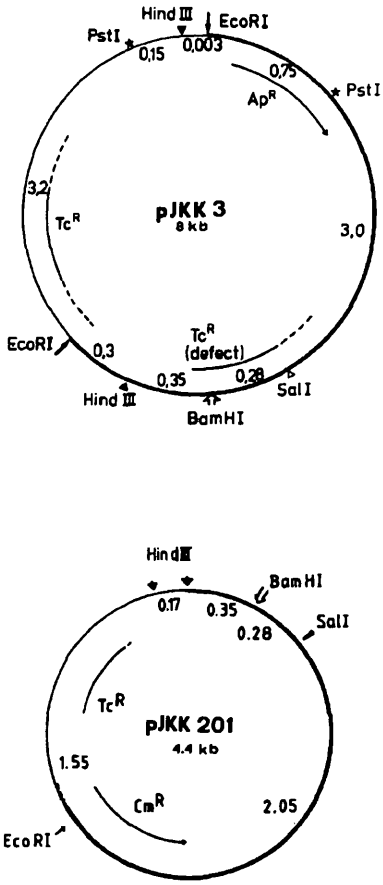

Fig. 1. Restriction maps of pJKK3 and pJKK201 for *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I and *Pst*I. pJKK3 was constructed by ligating *Hin*dIII linearized pBR322 and PBSU161-1 (Kreft et al. 1978). The construction of pJKK201 is described in the text. Fragment sizes are given in kilobase-pairs (kb)

performed with total RNA from *B. subtilis* BR151 carrying the plasmid under study. The RNA was purified as described above. After ethanol precipitation the RNA was dissolved in 30 µl hybridization buffer (Berk and Sharp 1977) to a concentration of 6.6 mg/ml. Fifteen µl of RNA (100 µg) were mixed with 5 µl of ^{32}P-end-labeled DNA fragment (0.5 µg DNA, 5×10^5 cpm/µg). After 15 min at 70° C hybridization was carried out at 53° C for 4 h. The mixture was then cooled in ice, diluted 10-fold with S1 nuclease buffer and 37.5 units of S1 nuclease (BRL) were added. After 30 min at 37° C the hybrids were precipitated by ethanol and either applied directly to DNA sequencing gels (6% polyacrylamide, 31% urea) or denatured in alkali (Green and Roeder 1980) prior to electrophoresis.

Elution of DNA Fragments. Restriction fragments of plasmid DNA were eluted from agarose gels by electroelution in dialysis bags using 10 mM Tris-acetate buffer pH 8.0 in the elution chamber (4 mA/cm, 45 min).

DNA Sequencing. Restriction fragments from plasmid DNA were cloned into M13mp8 according to Messing and Vieira (1982). Single-stranded recombinant DNA was isolated from phage and the nucleotide sequence of the inserted DNA was determined by the chain-termination method (Sanger et al. 1977) using a synthetic oligonucleotide as primer (gift of P.H. Seeburg, Genentech).

Results

Expression of Ampicillin Resistance

As previously reported, hybrid plasmids consisting of the *E. coli* vector pBR322 (Bolivar et al. 1977) and different parts of the *B. subtilis* plasmid pBSU161 have been constructed in vitro (Kreft et al. 1978). Plasmid pJKK3 (Fig. 1), one of several which replicate in *E. coli* and *B. subtilis*, determines resistance to tetracycline and ampicillin in *E. coli*. However, ampicillin resistance was not expressed when this plasmid was transformed into *B. subtilis*. No beta-lactamase activity could be detected intracellularly. A comparison of the proteins synthesized in minicells of *E. coli* and *B. subtilis* carrying pJKK3 or a derivative of this plasmid, where the beta-lactamase gene has been deleted in vitro, revealed that beta-lactamase and its precursor form were only synthesized in *E. coli*, and not in *B. subtilis* (Kreft et al. 1982). In order to localize the block in the expression of the beta-lactamase (*bla*) gene in *B. subtilis* we investigated next the in vivo transcription of this gene in *B. subtilis*. For this, RNA was purified from *B. subtilis* BR151 containing pJKK3 and dot blot hybridization (Kafatos et al. 1979) was carried out using the ^{32}P-labeled small *Eco*RI/*Pst*I fragment of pBR322, which contains most of the *bla* gene, as a gene specific probe. Figure 2 shows that no hybridization was obtained, indicating that the *bla* gene originating from pBR322 is not transcribed in *B. subtilis*. Deletions which may occur in pJKK3 during or subsequent to transformation into *B. subtilis* were not responsible for the lack of expression of the *bla* gene since this gene was fully functional upon retransformation of *E. coli* with plasmid DNA isolated from *B. subtilis*.

Expression of Chloramphenicol Resistance

A recombinant plasmid constructed in vitro from the *E. coli* vector pACYC184 (Chang and Cohen 1978) and pBSU161-1 (Kreft et al. 1978), lead in *E. coli* to the formation of pJKK201 (Fig. 1). The plasmid was apparently generated in vivo by an extensive deletion of the primary ligation product and expressed tetracycline and chloramphenicol resistance in *E. coli* (Kreft et al. 1978, 1982). *B. subtilis* could be transformed with pJKK201 to tetracycline resistance but not to chloramphenicol resistance. Plasmid DNA isolated from *B. subtilis* BR151 transformed with pJKK201 could retransform *E. coli* to chloramphenicol resistance. This indicates that the gene for chloramphenicol acetyltransferase (*cat*) is maintained in *B. subtilis* without rendering these cells resistant to chloramphenicol. Restriction analysis of the plasmid DNA isolated from *B. subtilis* transformed with pJKK201 also showed that the *cat* gene was structurally intact even in derivatives of pJKK201 which have been deleted in vivo in *B. subtilis* (results not shown).

RNA was isolated from *B. subtilis* BR151 containing pJKK201 and dot blot hybridization was carried out using the ^{32}P-labeled small *Hin*dIII/*Pst*I fragment of pBR325 (Bolivar 1978) as a probe-specific for *Tn*9-related *cat* genes. Figure 2 shows that no hybridization was obtained. This demonstrates that the *cat* gene from pACYC184 was also not transcribed in *B. subtilis*.

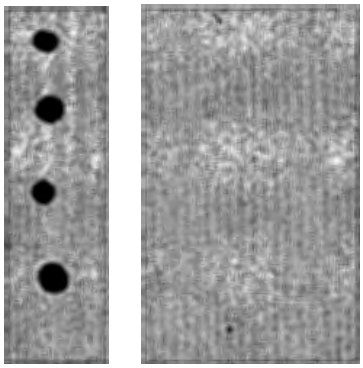

Fig. 2. Dot blot hybridization of RNA from *B. subtilis* carrying pJKK3 (b) or pJKK201 (c) with gene specific DNA probes. (a) control with pBSU161-1 DNA as a probe. From top to bottom 25, 50, 75 and 100 µg of RNA were applied to the filters

Expression of Tetracycline Resistance

It has been previously shown that the tetracycline resistance encoded by the *Bacillus* plasmid pBSU161-1 was expressed in *E. coli* (Kreft et al. 1978). We have also shown that a bifunctional plasmid consisting of the cryptic *B. subtilis* plasmid pBSU1 (Bernhard et al. 1978) and the *E. coli* vector pBR322 (Bolivar et al. 1977) could transform *B. subtilis* to tetracycline resistance (Goebel et al. 1979). In order to investigate further the expression of this *E. coli* derived tetracycline resistance determinant in *B. subtilis* two other recombinant plasmids were constructed in vitro.

pJKK310 consists of pBR325 (Bolivar 1978) and pUB110 (Gryczan et al. 1978) linearized by *Eco*RI and ligated subsequently with T4 DNA ligase (Fig. 3). This plasmid expressed resistance to ampicillin, kanaycin and tetracycline in *E. coli*. pJKK523 was constructed by ligating *Eco*RI linearized pBR322 with the staphyloccocal chloramphenicol resistance plasmid pC221 (Ehrlich 1977) which was also linearized by *Eco*RI (Fig. 3). After transformation of *E. coli* with the ligation mixture, colonies were selected which were resistant to ampicillin, chloramphenicol and tetracycline. *B. subtilis* protoplasts could be transformed by pJKK310 to kanamycin resistance or tetracycline resistance using DM3 regeneration plates (Chang and Cohen 1979) containing 100 µg/ml kanamycin or 15 µg/ml tetracycline, respectively. pJKK523 could transform *B. subtilis* to chloramphenicol resistance or tetracycline resistance using 7.5 µg/ml chloramphenicol or 15 µg/ml tetracycline in the selective regeneration medium. Most of the transformants obtained on single-selective agar plates did not express the second resistance marker present on the parental plasmid isolated from *E. coli*. Attempts to obtain transformants with these plasmids on double-selective regeneration plates failed. All of the single-resistant transformants contained plasmids which had suffered large deletions. Tetracycline resistant clones of *B. subtilis* obtained after transformation with pJKK523 harbored a deleted plasmid which contained most of the pBR322 sequence; their chloramphenicol resistant counterparts also contained a plasmid smaller than pJKK523 (results not shown). After subcultivation of the chloramphenicol resistant transformants no plasmid DNA could be isolated from these still chloramphenicol resistant strains, suggesting that the plasmid or part of it had become integrated into the chromosome. This shows that both

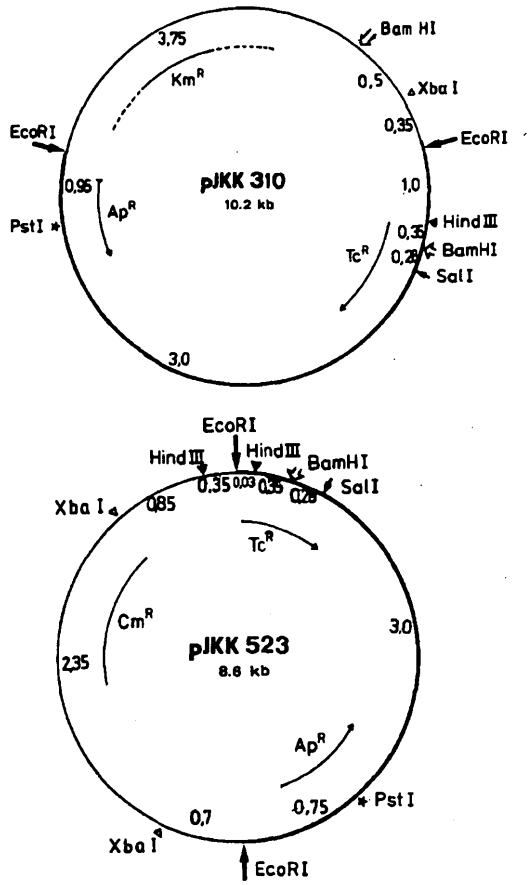

Fig. 3. Restriction maps of pJKK310 and pJKK523 for *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, *Pst*I and *Xba*I. The construction of the plasmids is described in the text. Fragment sizes are given in kb

pJKK310 and pJKK523 are structurally unstable in *B. subtilis*.

Only a few percent of the transformants obtained with these plasmids expressed both resistance markers, i.e. kanamycin plus tetracycline resistance or chloramphenicol plus tetracycline resistance in *B. subtilis*. All these transformants contained plasmid DNA which was indistinguishable from the parental plasmids by restriction analysis (data not shown).

These results indicate that the tetracycline resistance gene (*tet*) of pBR322 or pBR325 was expressed in *B. subtilis* in contrast to the two other antibiotic resistance genes mentioned above.

Next, we determined if the transcription of this *tet* gene starts in *B. subtilis* at its original (*E. coli*) promoter in these hybrid replicons or whether transcription of the tetracycline resistance gene is mediated by a read-through transcript starting at a promoter in the *S. aureus* part of the recombinant plasmids. In pJKK310 the *tet* gene is about 1,000 bp away from the junction site between the two parental plasmids; in pJKK523 this distance is only 30 bp.

By S1 nuclease mapping (Berk and Sharp 1977) we analyzed the transcripts from the *E. coli tet* gene which were synthesized in *B. subtilis* BR151 carrying intact pJKK310 or pJKK523, respectively. Total RNA was isolated from these strains and hybridized to suitable restriction fragments of the plasmids which were end-labeled with ^{32}P. In the case of pJKK310 the plasmid was cut by *Bam*HI, labeled with ^{32}P-γ-ATP and T4 polynucleotide kinase and

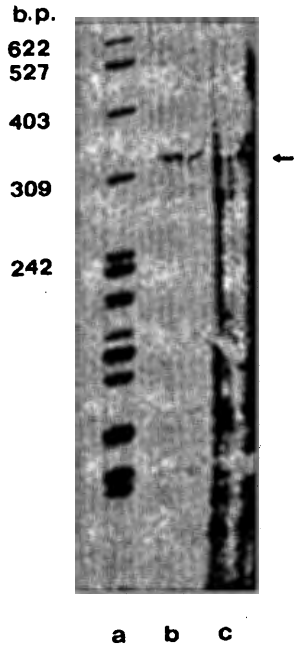

Fig. 4. S1 nuclease mapping of the 5′ termini of in vivo synthesized *tet*-gene mRNA. (a) *Hpa*II digest of pBR322, end-labeled with ^{32}P-γ-ATP. The size in base pairs (bp) of the largest fragments is indicated on the left. (b) and (c) RNA/DNA hybrids obtained after S1 nuclease treatment of fragments of pJKK310 (b) or pJK523 (c) hybridized with in vivo synthesized RNA from *B. subtilis* containing pJKK310 or pJKK523, respectively. The arrow indicates the largest transcript found, the smaller bands presumably represent prematurely terminated or degraded transcripts

then digested with *Eco*RI. The fragments were separated by electrophoresis on agarose gels. The *Bam*HI/*Eco*RI fragment upstream from the *Bam*HI site in the tetracycline resistance gene was isolated from the gel and used as a hybridization probe.

The transcription from pJKK523 was analyzed by using the *Bam*HI/*Xba*I fragment containing the presumed start site of the *tet* gene for the hybridization. Treatment of the DNA/RNA-hybrids with S1 nuclease and subsequent separation on denaturing polyacrylamide/urea gels showed (Fig. 4) that the longest transcript obtained in vivo with both plasmids had a size of about 330 bases. This size is very close to the expected size for a transcript starting at the original promoter of the *E. coli* tetracycline resistance determinant (*tet* promoter).

To obtain further evidence that the original *E. coli tet* promoter functions in *B. subtilis*, a derivative of pJKK310 was constructed. pJKK310 was linearized with *Hin*dIII which cuts within the −10 region of the *tet* promoter. The sticky ends were filled in using AMV reverse transcriptase (James et al. 1982) and blunt-end ligated by TE4 DNA ligase. *E. coli* was transformed with the ligation mixture, kanamycin resistant and tetracycline sensitive transformants were selected. One of these contained a plasmid, designated pJKK310 *ΔHin*d, which was identical to pJKK310 except that the *Hin*dIII site which is located in the *tet* promoter was destroyed by insertion of four base pairs, thus changing the distance between the −35 and −10 regions of the *tet* promoter. It has been previously demonstrated that slight changes in this distance strongly affect the promoter activity in *E. coli* (Russel and Bennett 1982). *B. subtilis* protoplasts could be transformed by pJKK310 *ΔHin*d to kanamycin resistance, transformation to tetracycline resistance failed. In this case all the kanamycin resistant and tetracycline sensitive transformants contained a plasmid indistinguishable from pJKK310 *ΔHin*d (data not shown). This clearly indicates that inactivation of the *E. coli tet* promoter also prevented the expression of the tetracycline resistance in *B. subtilis* and further suggests that the *tet* gene from pBR325 (which is identical to that of pBR322) was transcribed from its own promoter in *B. subtilis*. The possibility that a frame-shift caused by the four base pair insertion at the original *Hin*dIII site resulted in the formation of an altered and inactive fusion protein transcribed and translated from the *S. aureus* part of pJKK310 can be ruled out since there are several translation-stop codons in the pBR325 sequence preceeding the *tet* promoter (positions 12, 29, 36, 44, 58, 66 in pBR322) in all three reading frames.

To see if any mutation(s) occured in the *tet* promoter region of pJKK310 after transformation of the plasmid into *B. subtilis* this region was sequenced and compared to the published sequence of pBR325 (Prentki et al. 1981). The nucleotide sequence of the *tet* promoter region of pJKK310 was found to be identical with that of pBR325 (Fig. 5).

Discussion

The three *E. coli* genes investigated in this study which express resistance to ampicillin (*bla*), chloramphenicol (*cat*) and tetracycline (*tet*) are well characterized. The sequence of the structural genes and their transcription control regions in *E. coli* was determined (Sutcliffe 1979; Alton and Vapnek 1979). In addition the promoters and the initiation points of the in vitro transcripts have been mapped (Le Grice and Matzura 1981; Russel and Bennett 1981; Stüber and Bujard 1981).

```
                TTGACA                TATAAT
                -35                   -10
ACCGCAAGCGACAGGCCATGTTTGACAGCTTATCATCGATAAGCTTTAATGCG  tet pJKK310 (pBR325)
TTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAAC  bla pJKK3   (pBR322)
GAAAATGAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAA  cat pJKK201 (pACYC184)

TGAAAAATTTGCAAAAAGTTGTTGACTTTATCTACAAGGTGTGGCATAATAAT  SPO1-26
TAAAAATTTTACAAAAAGGTATTGACTTTCCCTACAGGGTGTGTAATAATTTA  SPO1-15

ATTTAACGATCACTCATCATGTTCATATTTATCAGAGCTCGTGCTATAATTAT  pE194 29k leader
TATATTTATGTTACAGTAATATTGACTTTTAAAAAAGGATTGATTCTAATGAA  pC194 cat
```

Fig. 5. Comparison of promoter regions. Apart from the pJKK310 *tet*-promoter the other sequences are derived from published information, the references are given in the text. The consensus sequences in the −35 and −10 regions of *B. subtilis* vegetative promoters are shown on top. The −35 and −10 regions of the promoters discussed here are overlined, sequences homologous to the consensus sequences are underlined, those with additional homology to SPO1 promoters are indicated by broken lines

The transcriptional mechanism of *B. subtilis* is more complex than that of *E. coli* (Losick and Pero 1981). The similarity between *E. coli* promoters and *B. subtilis* vegetative promoters (Lee and Pero 1981; Losick and Pero 1981; Moran et al. 1982) may suggest that the lack of expression of *E. coli* genes in *B. subtilis* is not caused by differences in these sequences.

Our data presented here, however, suggest that the RNA polymerase of *B. subtilis* is apparently unable to transcribe the *E. coli* genes determining ampicillin resistance (*bla*) and chloramphenicol resistance (*cat*) on the plasmids pBR322 and pACYC184 when they are introduced into *B. subtilis* via recombinant plasmids. From these data it cannot be ruled out that mRNAs transcribed from *E. coli* genes are extremely unstable in *B. subtilis*. However, the successful expression of the tet gene from *E. coli* in this host indicates that this is not the case. In contrast to the *E. coli bla* and *cat* genes the determinant for tetracycline resistance (*tet*) on pBR322 and pBR325 is expressed in *B. subtilis*. In *E. coli* transcription of the *tet*-gene starts about 335 base pairs upstream from the *Bam*HI site on pBR322 (West and Rodriguez 1982; J. Lowrie and J. Hedgpeth, personal communication). The longest transcript synthesized in *B. subtilis* from the corresponding part of the recombinant plasmids pJKK310 and pJKK523 as determined by S1 nuclease mapping is about 330 bases in size. This strongly suggests that the *tet* gene is transcribed in *B. subtilis* from its original *E. coli* promoter. From these results and those obtained with the related plasmid pJKK310Δ*Hin*d, where the *E. coli tet* promoter has been destroyed, it can be ruled out that transcription of the *tet* gene starts at a promoter in the *S. aureus* part of the hybrid plasmids.

If one compares the consensus sequences in the -35 and -10 regions (TTGACA and TATAAT, respectively) of *B. subtilis* vegetative promoters (Losick and Pero 1981) which are recognized by the initiation factor σ^{55}, with the corresponding sequences of the *E. coli bla*, *cat* and *tet* promoters one observes a striking homology between the *B. subtilis* and the *E. coli tet* promoters with only one mismatch in the -10 region (Fig. 5). On the other hand the promoter sequence for the *bla* gene shows two mismatches in both the -35 and -10 regions with three purine/pyrimidine nucleotide substitutions. The *cat* promoter has hardly any homology in the -35 region, the sequence with maximum homology having four mismatches with three purine/pyrimidine nucleotide substitutions; the -10 region has one mismatch, in addition the distance between the -35 and -10 regions is 20 base pairs instead of the 17 base pairs found with the other promoters (Fig. 5).

The *B. subtilis* phage SPO1 early genes SPO1-15 and SPO1-26 show conserved oligo-(dA) and oligo-(dT) sequences upstream from the -35 region (Lee and Pero 1981) (Fig. 5). It has been quoted that such sequences may be essential for *B. subtilis* vegetative promoters (Murray and Rabinowitz 1982; S. Chang, 4th International Symposium on Genetics of Industrial Microorganisms, Kyoto 1982). However, neither the *E. coli tet* promoter nor the promoters of the 29 k leader peptide from pE194 (Horinouchi and Weisblum 1980; Gryczan et al. 1980) which initiates transcription of the erythromycin resistance gene, and of the pC194 *cat* gene (Horinouchi and Weisblum 1982) show such sequences (Fig. 5). Nevertheless all three genes, which only show homology in their -35 and -10 regions, are readily expressed in *B. subtilis* (Ehrlich 1977; Weisblum et al. 1979).

Therefore it seems likely that the degree of homology in the -35 and -10 promoter regions of heterolous genes with the consensus sequence for *B. subtilis* vegetative (σ^{55}) promoters is essential for the expression in vegetative *B. subtilis* cells. Additional sequence homologies in other parts of the promoter region may well have an influence on the promoter efficiency in this bacterium, in which, apparently, more stringent sequence requirements have to be met by promoters than in *E. coli*.

Acknowledgements. The authors wish to thank M. Vogel for her expert technical assistance, A. Juarez for performing a large portion of the DNA sequencing, I. Grummt for advice on S1 nuclease mapping, M.S. Gilmore for reading of the manuscript and K.F. Fischbach for help during the computer editing of it. J. Lowrie and J. Hedgpeth are thanked for communicating results prior to publication.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 105 – A11).

References

Alton NK, Vapnek D (1979) Nucleotide sequence analysis of the chloramphenicol resistance transposon *Tn*9. Nature 282:864–869

Berk AJ, Sharp PA (1977) Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell 12:721–732

Bernhard K, Schrempf H, Goebel W (1978) Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. J Bacteriol 133:897–903

Bolivar F (1978) Construction and characterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant molecules. Gene 4:121–136

Bolivar F, Rodriguez RL, Greene PJ, Betlach MC, Heyneker HL, Boyer HW, Crosa JH, Falkow S (1977) Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113

Chang ACY, Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 134:1141–1156

Chang S, Cohen SN (1979) High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol Gen Genet 168:111–115

Cohen SN, Chang ACY, Boyer HW, Helling RB (1973) Construction of biologically functional plasmids in vitro. Proc Natl Acad Sci USA 70:3240–3244

Cruz de la F, Müller D, Ortiz JM, Goebel W (1980) Hemolysis determinant common to *Escherichia coli* hemolytic plasmids of different incompatibility groups. J Bacteriol 143:825–833

Dubnau D, Gryczan TJ, Contente S, Shivakumar AG (1980) Molecular cloning in *Bacillus subtilis*. In: Setlow JK, Hollaender A (eds) Genetic engineering. Plenum Press, New York, pp 115–131

Ehrlich SD (1977) Replication and expression of plasmids from *Staphylococcus aureus* in *Bacillus subtilis*. Proc Natl Acad Sci USA 74:1680–1682

Ehrlich SD (1978) DNA cloning in *Bacillus subtilis*. Proc Natl Acad Sci USA 75:1433–1436

Ehrlich SD, Niaudet B, Michel B (1982) Use of plasmids from *Staphylococcus aureus* for cloning of DNA in *Bacillus subtilis*. Current Topics Microbiol Immunol 96:19–29

Glisin V, Crkvenjakov R, Byus C (1974) Ribonucleic acid isolation by cesium chloride centrifugation. Biochemistry 13:2633–2637

Goebel W, Bonewald R (1975) Class of small multicopy plasmids originating from the mutant antibiotic resistance factor R1*drd*-19B2. J Bacteriol 123:658–665

Goebel W, Kreft J, Burger KJ (1979) Molecular cloning in *Bacillus subtilis*. In: Timmis KN, Pühler A (eds) Plasmids of medical, environmental and commercial importance. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 471–480

Goldfarb DS, Doi RH, Rodriguez RL (1981) Expression of Tn9-derived chloramphenicol resistance in *Bacillus subtilis*. Nature 293:309–311

Goldfarb DS, Rodriguez RL, Doi RH (1982) Translational block to expression of the *Escherichia coli Tn*9-derived chloramphenicol resistance gene in *Bacillus subtilis*. Proc Natl Acad Sci USA 79:5886–5890

Green MR, Roeder RG (1980) Definition of a novel promoter for the major adenovirus-associated virus mRNA. Cell 22:231–242

Gryczan TJ, Dubnau D (1978) Construction and properties of chimeric plasmids in *Bacillus subtilis*. Proc Natl Acad Sci USA 75:1428–1432

Gryczan TJ, Contente S, Dubnau D (1978) Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J Bacteriol 134:318–329

Gryczan TJ, Grandi G, Grandi R, Dubnau D (1980) Conformational alteration of mRNA structure and the post-transcriptional regulation of erythromycin-induced drug resistance. Nucleic Acids Res 8:6081–6097

Horinouchi S, Weisblum B (1980) Post-transcriptional modification of mRNA conformation: Mechanism that regulates erythromycin-induced resistance. Proc Natl Acad Sci USA 77:7079–7083

Horinouchi S, Weisblum B (1982) Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J Bacteriol 150:815–825

James AA, Morrison PT, Kolodner R (1982) Genetic recombination of bacterial plasmid DNA. Analysis of the effect of recombination-deficient mutations on plasmid recombination. J Mol Biol 160:411–430

Kafatos FC, Jones CW, Efstradiadis A (1979) Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res 7:1541–1552

Kreft J, Bernhard K, Goebel W (1978) Recombinant plasmids capable of replication in *B. subtilis* and *E. coli*. Mol Gen Genet 162:59–67

Kreft J, Hughes C (1982) Cloning vectors derived from plasmids and phage of *Bacillus*. Current Topics Microbiol Immunol 96:1–17

Kreft J, Parrisius J, Burger KJ, Goebel W (1982) Expression and instability of heterologous genes in *B. subtilis*. In: Ganesan AT, Chang S, Hoch JA (eds) Molecular cloning and gene regulation in *Bacilli*. Academic Press, New York, pp 145–157

Lee G, Pero J (1981) Conserved nucleotide sequences in temporally controlled bacteriophage promoters. J Mol Biol 152:247–265

Le Grice SFJ, Matzura H (1981) Binding of RNA polymerase and the catabolite gene activator protein within the *cat* promoter in *Escherichia coli*. J Mol Biol 150:185–196

Losick R, Pero J (1981) Cascades of sigma factors. Cell 25:582–584

Lovett PS, Keggins KM (1979) *Bacillus subtilis* as a host for molecular cloning. In: Wu R (ed) Methods in microbiology, vol 68. Academic Press, New York, pp 342–357

Mayer H, Luibrand G, Goebel W (1977) Replication of the mini-R1 plasmid Rsc11 and Rsc11 hybrid plasmids. Mol Gen Genet 152:145–152

Messing J, Vieira J (1982) A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19:269–276

Meyers J, Sanchez D, Elwell L, Falkow S (1975) Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J Bacteriol 127:1529–1537

Moran CP Jr, Lang N, Le Grice SFJ, Lee G, Stephens M, Sonenshein AL, Pero J, Losick R (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol Gen Genet 186:339–346

Murray CL, Rabinowitz JC (1982) Nucleotide sequences of transcription and translation initiation regions in *Bacillus* phage ϕ29 early genes. J Biol Chem 257:1053–1062

Prentki P, Karch F, Iida S, Meyer J (1981) The plasmid cloning vector pBR325 contains a 482 base-pair-long inverted duplication. Gene 14:289–299

Russel DR, Bennett GN (1981) Characterization of the beta-lactamase promoter of pBR322. Nucl Acids Res 9:2517–2533

Russel DR, Bennett GN (1982) Construction and analysis of in vivo activity of *E. coli* promoter hybrids and promoter mutants that alter the -35 to -10 spacing. Gene 20:231–243

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467

Stüber D, Bujard H (1981) Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc Natl Acad Sci USA 78:167–171

Sutcliffe JG (1979) Complete nucleotide sequence of the *Escherichia coli* plasmid pBR322. Cold Spring Harbor Symp Quant Biol 43:77–90

Weisblum B, Graham MY, Gryczan TJ, Dubnau A (1979) Plasmid copy number control: Isolation and characterization of high-copy number mutants of plasmid pE194. J Bacteriol 137:635–643

West RW Jr, Rodriguez RL (1982) Construction and characterization of *E. coli* promoter-probe plasmid vectors. II. pBR322 derivatives with deletions in the tetracycline resistance promoter region. Gene 20:291–304

Williams DM, Schoner RG, Duvall EJ, Preis LH, Lovett PS (1981) Expression of *Escherichia coli trp* genes and the mouse dihydrofolate reductase gene cloned in *Bacillus subtilis*. Gene 16:199–206

Wilson CR, Baldwin JN (1978) Characterization and construction of molecular cloning vehicles within *Staphylococcus aureus*. J Bacteriol 136:402–413

Communicated by E. Bautz

Received February 23, 1983