Volume 15 Number 6 1987 Nucleic Acids Research

Activity of a *Streptomyces* transcriptional terminator in *Escherichia coli*

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Received January 20, 1987; Revised and Accepted February 25, 1987

ABSTRACT

A 205bp DNA fragment from the Streptomyces multi-copy plasmid pIJ101 has in vivo terminator activity both in Streptomyces lividans and in Escherichia coli. Termination of RNA synthesis, detected by high-resolution S1 nuclease mapping, occurs at precisely the same nucleotides in both organisms. This suggests that the E. coli RNA polymerase recognizes the same sequence elements and chooses the point(s) of termination in the same way as the corresponding S. lividans enzyme.

INTRODUCTION

Streptomyces lividans and Escherichia coli are two taxonomically very distant bacteria. For some practical purposes it is advantageous to manipulate Streptomyces DNA fragments cloned in E. coli. Much attention has been given to the expression of Streptomyces promoters in E. coli and vice versa. E. coli promoters are generally recognized and well utilized by S. lividans, whereas only few Streptomyces promoters are active in E. coli [1-3]. There are several possible reasons for this. One important factor may be that, under "normal" circumstances, only one RNA polymerase holoenzyme form is active in E. coli while Streptomyces has at least three holoenzymes, one of them recognizing E. coli-like promoters [4,5]. A second important consideration is the unusual base composition of Streptomyces DNA (c. 73% G+C residues): the high G+C content of DNA surrounding the promoters of Streptomyces genes may be a barrier to transcription in E. coli [4,6].

Termination of transcription may also be important for the regulation of gene expression. Thus, many genes are regulated by attenuation [7], while differential expression of genes in the same operon may be achieved by the presence of weak terminators within the operon. Special anti-termination mechanisms are involved in the life cycle of phage lambda and in the regulation of ribosomal RNA synthesis in E. coli (reviewed by Platt [8] and Morgan [9], respectively).

Terminators in E. coli are classified as rho-dependent or rho-independent. Rho-independent termination often occurs at a series of uridine residues after a hairpin structure has been formed by the nascent RNA molecule. However, not all rho-independent terminators fit this generalization. In contrast, no consensus structure or sequence has been derived from the known sequences of rho-dependent terminators. The study of transcription termination is complicated by the fact that genuine ends of transcripts cannot be distinguished unambiguously from ends generated by RNA processing or degradation. In vitro studies prevent this problem but they do not always reflect the in vivo situation faithfully. (See Platt [8] and references therein for reviews on these topics.)

Little is so far known about transcript termination in Streptomyces. The gyl "0.9kb" transcript of S. coelicolor was found by low-resolution S1 mapping to end shortly after an inverted repeat [10]. The termination point of the transcript for the hyg (hygromycin phosphotransferase) gene of S. hygroscopicus was determined by high resolution S1 mapping [11]. The sequences of the Streptomyces aph, tsr and vph genes contain inverted repeats which might be transcriptional terminators [12, 13]. None of these inverted repeats in the DNA sequence is followed by a run of thymidines (uridines in the RNA).

Several E. coli terminators have been shown to function in vivo in S. lividans but none of the termination points has yet been shown to be identical to those in E. coli. They include the major terminator from phage fd [14], the ampC terminator from the E. coli chromosome [2] and two terminators from phage lambda, tR1 [15] and toop (T. Eckhardt, personal communication).

Here we describe the sequence of a DNA fragment from the wide host-range Streptomyces plasmid pIJ101 [16] which has in vivo terminator activity both in S. lividans and in E. coli. Termination, detected by S1 nuclease mapping, occurs in both organisms at the same nucleotides. The transcripts which end at this terminator originate from the promoters Pc [17] and Pd and are involved in the control of pIJ101 copy-number and incompatibility (Deng et al., in preparation).

MATERIALS AND METHODS

Bacterial strains and plasmids. TK24 (str-6) and TK64 (str-6, pro-6) are plasmid-free derivatives of S. lividans 66 [18]. TK146 is TK24 containing pIJ101 [16]. The Streptomyces terminator-probe plasmid pIJ459 and the promoter-probe plasmids pIJ486 and pIJ487 are derivatives of pIJ101 described

by Ward et al. [14]. E. coli strain ED8767 (metB, recA, hsdS) [19] was used as host for all the E. coli plasmids. The promoter-probe plasmid pKK232-8 [20] was used as cloning vector in E. coli. Strain DX31 is ED8767 containing pIJ2727 (Fig. 1). Other plasmids are shown in Figs. 1 and 2.

General techniques for the manipulation of bacteria and nucleic acids. Techniques for handling S. lividans and E. coli and for the manipulation of nucleic acids were described in detail by Hopwood et al. [21]. Plasmid isolation was by the method of Kieser [22].

Measurement of antibiotic resistance. The levels of antibiotic resistance (chloramphenicol for E. coli and kanamycin for S. lividans) were determined using antibiotic gradient plates [23] with L-agar [24] and MM supplemented with proline [21], respectively.

RNA isolation. RNA was isolated from 2d old cultures of S. lividans derivatives grown in YEME medium containing 34% sucrose and 5mM $MgCl_2$ and from E. coli grown in L-broth. The following modification of the published procedure [21] was necessary for strains containing multicopy plasmids. Steps 10 and 11 (normally alternatives) were used sequentially to remove DNA completely. After precipitation of DNA with 4M sodium acetate (step 10) the pellet was redissolved in distilled water, followed by digestion with RNase-free DNase (Worthington-DPFF) (step 11).

Preparation of the end-labelled probe DNA fragment for S1 protection experiments. The DNA probe labelled at the 3' end of the ApaI site in the BclI-E fragment of pIJ101 was prepared by digesting pIJ101 with ApaI (four sites) and the 3' ends were labelled with terminal deoxynucleotidyltransferase (BRL) and [α^{32}P]ddATP (Amersham) by the method of Yousaf et al. [25]. The labelled DNA was then digested with BglII (one site in pIJ101) and the 800bp fragment was isolated by agarose gel electrophoresis and electroelution.

Determination of the 3' ends of transcripts by S1 nuclease mapping. 50µg of total RNA was mixed with approximately 0.025pmol of labelled DNA (c. 10^5 cpm) and incubated at 85°C for 10min in hybridization solution [26]; the temperature was then lowered to 67°C and incubation continued for 6h. Hybridization reactions were diluted with 300µl of ice-cold S1 digestion buffer [26] containing 150 units of S1 nuclease (Type III, Sigma) and chilled on ice. S1 digestion was done at 37°C for 45min and ended by adding 25µl of termination solution [26]. S1-resistant hybrids were precipitated with 1 vol of isopropanol and analyzed on a 8% polyacrylamide gel containing 7M urea [27] followed by autoradiography at -70°C.

DNA sequencing. DNA was sequenced by the method of Maxam and Gilbert

[28] with the modifications specified in [21]. 3' end-labelled fragments were generated by digesting pIJ101 with BclI and filling in with [α^{32}P]dGTP (c. 3000Ci/mMol, New England Nuclear) and the Klenow fragment of DNA polymerase I (Pharmacia) according to the method of Maniatis et al. [29], followed by digestion with SstI. 5' XmaI ends were labelled after treatment with calf intestinal alkaline phosphatase (Boehringer) using [γ^{32}P]dATP (c. 3000Ci/mMol, New England Nuclear) and polynucleotide kinase (BRL) followed by digestion with BglII. Fragments labelled at only one end were purified by agarose gel electrophoresis. The sequence was determined from both strands of the DNA.

RESULTS AND DISCUSSION

Identification of a transcriptional terminator from the Streptomyces multicopy plasmid pIJ101 which functions in E. coli

The BclI-E fragment of the Streptomyces multi-copy plasmid pIJ101 contains two promoters which function in E. coli [17]. These promoters are indicated as Pc and Pd in Fig. 1. pIJ2727 (Fig. 1) contains the pIJ101 BclI-E fragment inserted into the BamHI site of the E. coli promoter-probe vector pKK232-8; this plasmid conferred resistance to 35μg/ml chloramphenicol. Deleting the 249bp fragment between the SmaI and the right hand BclI sites gave pIJ2791 (Fig. 1) which conferred resistance to more than 80μg/ml chloramphenicol. This finding indicates the existence of a terminator active in E. coli in the deleted segment. A plasmid (pIJ2788; Fig. 1) carrying the 249bp SmaI-BclI fragment alone conferred resistance to 7μg/ml chloramphenicol. (This level is significantly above the background of less than 3μg/ml obtained with pKK232-8 without insert, indicating the existence of a weak promoter active in E. coli in this fragment.)

We also subcloned the 205bp DpnI fragment (Fig. 1) into the SmaI site of pKK232-8. The resulting plasmid (pIJ2793) gave resistance to 7μg/ml chloramphenicol when the DpnI fragment was inserted in the original orientation (and less than 3μg/ml in the opposite orientation).

Demonstration of the terminator function in S. lividans

The DpnI fragment was excised from pIJ2793 by digestion with EcoRI and HindIII (which cut in the polylinker) and inserted into the Streptomyces promoter-probe plasmids pIJ486 and pIJ487. Resistance to less than 1μg/ml kanamycin (background level) was observed in S. lividans. The 205bp DpnI fragment was then excised from pIJ486 and pIJ487 by digestion with BglII and HindIII (which cut in the polylinker) and inserted into the Streptomyces

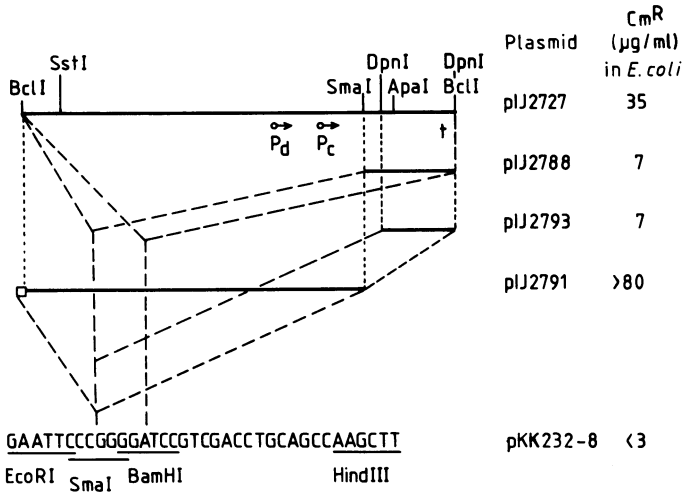

Figure 1. Segments of pIJ101 DNA that were cloned into the SmaI or BamHI sites of the E. coli promoter-probe plasmid pKK232-8. Only the sequence of part of the polylinker of pKK232-8 is shown; the promoterless cat gene is to the right of the HindIII site. pIJ2727 contains the whole BclI-E fragment of pIJ101 in the BamHI site of pKK232-8. Pc and Pd are the promoters described by Deng et al. [17] and t is the transcriptional terminator studied in this paper. pIJ2788 was constructed by digesting pIJ2727 with SmaI and religating. pIJ2793 contains the 205bp DpnI fragment cloned into the SmaI site of pKK232-8 and pIJ2791 was constructed by excising the 930bp SmaI fragment from pIJ2727 and cloning it into the SmaI site of pKK232-8; the open square indicates the linker sequence which was added to this fragment.

terminator-probe plasmid pIJ459 (Fig. 2) digested with BamHI and HindIII. pIJ459 without insert conferred resistance to 180μg/ml kanamycin on S. lividans. This resistance was reduced to 60μg/ml when the DpnI fragment was inserted in the original orientation (pIJ2615) and to 5μg/ml when it was present in the opposite orientation (pIJ2616). This indicates that the 205bp DpnI fragment can terminate transcription coming from both directions in S. lividans, and much more strongly in one orientation than in the other.

Determination by high-resolution S1 nuclease mapping of the 3' ends of the transcripts generated in S. lividans and in E. coli

RNA was isolated from S. lividans TK146 (containing pIJ101) and from E. coli DX31 (containing pIJ2727). RNA from plasmid-free strains served as controls. The 800bp ApaI-BglII probe DNA (see Materials and Methods) was labelled at the 3' end of the ApaI site (which is within the 205bp DpnI fragment: Fig. 1). Fig. 3 shows the result of an S1 mapping experiment using this probe. RNA from DX31 (lane 2) and from TK146 (lane 3) protected the same

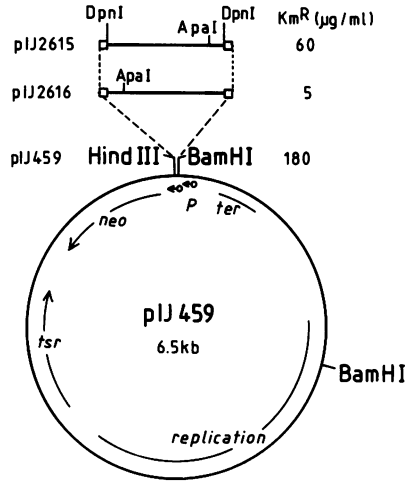

Figure 2. Insertion of the 205bp DpnI fragment into the Streptomyces terminator-probe plasmid pIJ459. The DpnI fragments from pIJ2793 (Fig. 1) and from pIJ2792 (identical to pIJ2793 except that the DpnI fragment was inserted into the SmaI site of pKK232-8 in the opposite orientation) were excised by digestion with EcoRI and HindIII and inserted into pIJ486 digested with the same enzymes. From there the fragments were excised by digestion with BglII and HindIII and inserted between the strong ermE-promoters (ermEP1 and ermEP2: [34]) and the promoterless neo gene of pIJ459. (The locations and the orientations of the two ermE-promoters are indicated with o→ and the letter P.) This was achieved by digesting pIJ459 with BamHI and HindIII. (Three fragments had to be ligated together to give a viable plasmid because pIJ459 contains two BamHI sites, one of them in the replication region.) The open squares at the ends of the DpnI fragments indicate the polylinker sequences carried over from pKK232-8 and from pIJ486. ter: transcriptional terminator from phage fd. tsr: thiostrepton resistance gene.

length of the probe DNA. As is commonly found, transcription seems to end at a series of positions lying within a few residues of each other rather than at a single residue. Six clustered bands with similar relative intensities in the two hosts can be seen, implying that RNA polymerases of the two organisms recognize the same sequence elements as terminators and choose the transcript end-points in the same way. The determination of transcriptional end-points is complicated by the general problem that the 3' ends of transcripts are indistinguishable from 3' ends generated by endo- or exonucleolytic cleavage. During RNA degradation specific fragments accumulate either because of endonucleolytic cleavage or through stalling of exonucleases at hairpin structures (reviewed by Higgins and Smith [30]). The homology between the probe fragment and the DNA sequence in pIJ2727, which was the template for the

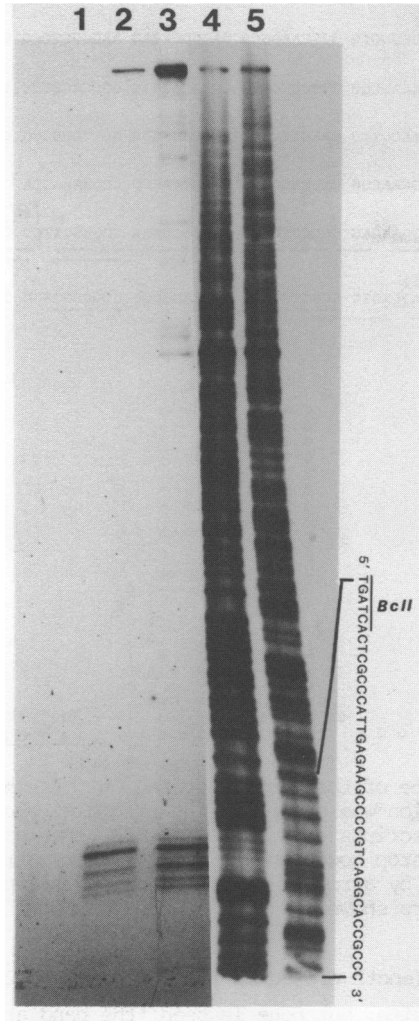

Figure 3. S1 nuclease mapping of the 3' end of the RNA synthesized in E. coli DX31 and in S. lividans TK146. The DNA probe was an 800nt fragment labelled at the 3' end of the ApaI site of the BclI-E fragment of pIJ101 (Fig. 1) and extending to the single BglII site in pIJ101. Lane 1: S1 protection with RNA from S. lividans TK24 (control). Lane 2: RNA from E. coli DX31 (containing pIJ2727). Lane 3: RNA from S. lividans TK146 (containing pIJ101). Lanes 4 and 5: sequence ladders from the strand complementary to the probe DNA. TC- and GA-specific cleavage, respectively (T's are weaker than C's). Lanes 1-3 were exposed longer than lanes 4 and 5.

RNA synthesis in E. coli DX31, extends only to the right hand BclI site (Fig. 3), 13bp past the main termination point (see below). This makes it possible to distinguish between full-length protection and DNA-DNA hybridization

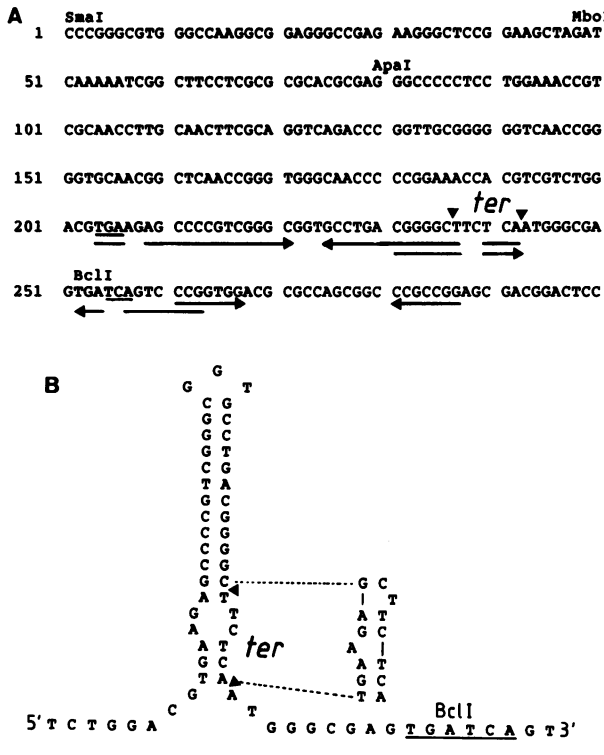

Figure 4. DNA sequence of the terminator region. A. ▼ter▼ indicates the region where termination was observed, the triangles showing where the largest and the smallest transcripts end. Relevant TGA translational stop sites are underlined (TCA is a stop codon on the complementary strand). Inverted repeats are indicated by arrows. B. The proposed hairpin structure; two possible alignments are shown.

artefacts. Any full-length protected fragment in lane 2 would run at the position of the BclI site, but none is seen (the band at the top of this lane is due to DNA-DNA hybridization). In lane 3 (RNA from TK146) longer transcripts could be detected and the prominent band at the top of the gel is probably due to both DNA-DNA hybridization and full length protection of the probe. This experiment suggests that termination is stronger in E. coli than in S. lividans.

DNA sequence of the terminator region

Fig. 4A shows the sequence of the terminator region analyzed using DNA from pIJ101 itself. The RNA end-points occur in the stem of a potential hairpin loop 16 to 21 nucleotides after the centre of the loop shown in Fig.

4B. The free energy of the loop structure is estimated to be about -36Kcal [31]. There are two other potential hairpin structures in the terminator region as shown by the pairs of inverted arrows in Fig. 4A. Because some of these sequences overlap, formation of the "central" hairpin would prevent the formation of the other two hairpins, one of which, shown in Fig. 4B, forms the putative terminator structure.

All the rho-independent (E. coli) terminators listed by Brendel et al. [32] lead to termination several nucleotides past the hairpin structure, usually after a series of U's has been added to the RNA. The base-pairing at the foot of our hypothetical RNA hairpin structure is destabilized by the adjacent loops and the hairpin structure formed in vivo could be smaller than that drawn in Fig. 4B. No row of U's precedes the termination point, but this feature is not always found in E. coli terminators and would be even more unusual in the G+C-rich Streptomyces DNA (the BclI-E fragment of pIJ101 has 71.6% G+C). The sequences CGGG(C/G) and TCTG are frequently found in E. coli terminators [32,33]; CGGG(C/G) is generally very frequent in Streptomyces DNA and occurs twice in the hairpin structure which precedes the termination points. The sequence TCTG, which often occurs downstream of the run of thymidine residues in E. coli terminators, does not occur in our sequence.

CONCLUSION

We cannot exclude the possibility that our sequence, rather than acting as a transcriptional terminator, is highly susceptible to endonucleolytic cleavage in vivo and thus mimics the function of a terminator both in the S1 protection experiment and in the experiments with the "terminator-probe" plasmids. It would be difficult to postulate, however, that endonucleolytic cleavage could inactivate the transcript because, if such cleavage occurred in the constructs used here, the product would be a truncated transcript still containing the ribosome binding site and translation would not necessarily be affected. Analysis of the region of pIJ101 beyond the end of the transcript revealed promoters reading from the opposite direction towards the terminator (Z.D., unpublished). The open reading frames on the transcripts on either side of the terminator end with UGA codons each near the beginning of the proposed stem-loop structure (Fig. 4). It seems logical that the convergent transcripts are separated by a terminator that limits the uncontrolled generation of complementary RNA's. This is consistent with the finding of bidirectional terminator activity in S. lividans. It could be that the transcripts starting at Pc and Pd which proceed through the terminator

interact by hybridization with the transcripts coming from the other direction. (The incomplete termination observed in the left-to-right orientation is not an artefact caused by the exceptionally strong ermE promoters used in the terminator-probe plasmid pIJ459 because it was also observed in the S1 mapping experiment with RNA from TK146, containing pIJ101.) Evidence for a bidirectional Streptomyces terminator has also been obtained for the methylenomycin gene cluster (R.J. Neal, personal communication). The overlapping inverted repeats in the pIJ101 terminator region are reminiscent of an attenuator.

ACKNOWLEDGEMENTS

We thank J. Brosius for the plasmid pKK232-8 and R.J. Neal, Q.S. Ma and G.F. Hong for critical reading of the manuscript. Z.D. was supported by a graduate studentship from the Chinese government. This work was supported by the Agricultural and Food Research Council and the John Innes Foundation via grants-in-aid to the John Innes Institute. Support was also received from the United Nations Development Programme/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases.

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REFERENCES

1. Bibb, M.J. and Cohen, S.N. (1982) Mol. Gen. Genet. 187, 265-277.
2. Jaurin, B. and Cohen, S.N. (1984) Gene 28, 83-91.
3. Jaurin, B. and Cohen, S.N. (1985) Gene 39, 191-201.
4. Westpheling, J., Ranes, M. and Losick, R. (1985) Nature (London) 313, 22-27.
5. Grossman, A.D. and Losick, R. (1986) RNA polymerase heterogeneity in bacteria. In Regulation of gene expression - 25 years on (eds. I.R. Booth and C.F. Higgins), p127-138. Cambridge University Press, Cambridge.
6. Bibb, M.J., Chater, K.F. and Hopwood, D.A. (1983) Developments in Streptomyces cloning. In Experimental manipulation of gene expression (ed. M. Inoue), p53-82. Academic Press, London and New York.
7. Jensen, K.F., Bonekamp, F. and Poulsen, P. (1986) TIBS 11, 362-365.
8. Platt, T. (1986) Ann. Rev. Biochem. 55, 339-372.
9. Morgan, E.A. (1986) J. Bacteriol. 168, 1-5.
10. Smith, C.P. (1986) Ph.D. thesis, University of East Anglia, Norwich.
11. Zalacain, M., González, A., Guerrero, M.C., Mattaliano, R.J., Malpartida, F. and Jiménez, A. (1986) Nucl. Acids Res. 14, 1565-1581.
12. Thompson, C.J. and Gray, G.S. (1983) Proc. Natl. Acad. Sci. 80, 5190-5194.
13. Bibb, M.J., Bibb, M.J., Ward, J.M. and Cohen, S.N. (1985) Molec. Gen. Genet. 199, 26-36.
14. Ward, J.M., Janssen, G.R., Kieser, T., Bibb, M.J. and Bibb, M.J. (1986) Mol. Gen. Genet. 203, 468-478.
15. Brawner, M.E., Auerbach, J.I., Fornwald, J.A., Rosenberg, M. and Taylor, D.P. (1985) Gene 40, 191-201.

16. Kieser, T., Hopwood, D.A., Wright, H.M. and Thompson, C.J. (1982) Mol. Gen. Genet. 185, 223-238.
17. Deng, Z., Kieser, T. and Hopwood, D.A. (1986) Gene 43, 295-300.
18. Hopwood, D.A., Kieser, T., Wright , H.M. and Bibb, M.J. (1983) J. Gen. Microbiol. 129, 2257-2269.
19. Murray, N.E., Brammar, W.J. and Murray, K. (1977) Mol. Gen. Genet. 150, 53-61.
20. Brosius, J. (1984) Gene 27, 151-160.
21. Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H. (1985) Genetic Manipulation of Streptomyces: A Laboratory Manual, John Innes Foundation, Norwich.
22. Kieser, T. (1984) Plasmid 12, 19-36.
23. Szybalski, W. (1952) Science 116, 46-48.
24. Lennox, E.S. (1955) Virology 1, 190-206.
25. Yousaf, A.I., Carroll, A.R. and Clarke, B.E. (1984) Gene 27, 309-313.
26. Favaloro, J., Treisman, R. and Kamen, R. (1980) Methods Enzymol. 65, 718-749.
27. Sanger, F. and Coulson, A.R. (1978) FEBS Lett. 87, 107-110.
28. Maxam, A.M. and Gilbert, W. (1980) Methods in Enzymol. 65, 499-560.
29. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
30. Higgins, C.F. and Smith, N.H. (1986) Messenger RNA processing, degradation and the control of gene expression. In Regulation of Gene Expression - 25 years on (eds. I.R. Booth and C.F. Higgins), p179-198. Cambridge University Press, Cambridge.
31. Tinoco, I., Borer, P.N., Dengler, B., Levine, M.D., Uhlenbeck, O.C., Crothers, D.M. and Gralla, J. (1973) Nature New Biology 246, 40-41.
32. Brendel, V., Hamm, G.H. and Trifonov, E.N. (1986) J. Biomol. Struct. Dynam. 3, 705-723.
33. Brendel, V. and Trifonov, E.N. (1984) Nucl. Acids Res. 12, 4411-4427.
34. Bibb, M.J., Janssen, G.R. and Ward, J.M. (1986) Gene 41, E357-E368.