Nucleic Acids Research, Vol. 19, No. 16 4399–4403

A comparative study of the ribosomal RNA operons of *Streptomyces coelicolor* A3(2) and sequence analysis of *rrnA*

G.P.van Wezel, E.Vijgenboom+ and L.Bosch*
Department of Biochemistry, University of Leiden, PO Box 9502, 2300 RA Leiden, The Netherlands

Received May 31, 1991; Revised and Accepted July 19, 1991 EMBL accession no. X60514

ABSTRACT

S.coelicolor A3(2) contains six ribosomal RNA operons. Here we describe the cloning of _rrnA_, _rrnC_ and _rrnE_, thereby completing the cloning of all operons. Southern hybridisation of genomic DNA with a heterologous probe from the _E.coli rrnB_ 16S rRNA gene showed differences in hybridisation among the six rRNA operon-containing bands. The nucleotide sequence of the 16S rRNA gene and the upstream region of _rrnA_ was determined and compared with the corresponding sequence of _rrnD_, showing that the 16S rRNA genes are 99% identical. Substantial differences were found, however, in the upstream regions corresponding to the P1 and P2 promoters of _rrnD_. Southern analysis showed that some of the other rRNA operons of _S.coelicolor_ A3(2) also differed in this part of the upstream region.

INTRODUCTION

At present there is much interest in the genus *Streptomyces*, a group of filamentous, gram positive eubacteria (1). This interest stems largely from the complex life-cycle of the bacteria, which involves three stages of differentiation (2) and from their ability to produce a wide variety of secondary metabolites, including antibiotics (3), which confers considerable biotechnological importance. To fully understand these phenomena, knowledge about growth control in streptomycetes may prove essential.

In *E.coli* the expression of rRNA and tRNA plays a dominant role in the regulation of growth (4,5). Not much is known about these regulation processes in *Streptomyces spp*. To shed more light on these questions, a structural analysis of the rRNA gene sets and their regulatory sequences is a prerequisite.

The organisation of rRNA operons has recently been investigated in several *Streptomyces species*. *S.coelicolor* (6), *S.lividans* (7), *S.griseus* (8) and *S.rimosus* (9) contain six ribosomal RNA operons, whereas *S.ambofaciens* (10) contains only four. In all streptomycetes studied thus far the gene order is 16S–23S–5S rRNA. In *E.coli*—like most microorganisms—the spacer region between the 16S and the 23S rRNA genes contains a tRNA gene (11), but in *Streptomyces spp* this is not the case (6,7,10). *Mycobacterium bovis*, an organism closely related to *Streptomyces spp*, also lacks a tRNA gene in the spacer region (12) suggesting that this is typical for filamentous bacteria.

The entire sequence of the *S.ambofaciens rrnD* operon has been elucidated (10), whereas only partial sequences of other investigated streptomycete rRNA operons have been published. In the case of the *S.coelicolor* A3(2) *rrnD* operon, the sequence of the 16S rRNA gene and flanking regions was published (13,14).

Interestingly, a preliminary survey suggested sequence differences among the rRNA operons of *S.coelicolor*. It provided the impetus for the present study, which is a basis for our future research on transcription regulation of the *S.coelicolor* rRNA operons. Here we analyse possible differences among the operons of this organism. We describe the cloning of three of the operons (*rrnA*, *rrnC* and *rrnE*) and analyse the sequence of the 16S rRNA gene and upstream region of *rrnA*.

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages

S.coelicolor A3(2) M145 (15) was obtained from the John Innes Institute in Norwich (England) and was cultured in YEME (15). Plasmid pBR329 (16) and bacteriophage M13 derivatives (17) were propagated in *E.coli* strains JM101 (18) and JM109 (19) and λ phages in *E.coli* LE392 (20). All *E.coli* strains were cultured in LB medium (8g Difco Bactotryptone, 5g NaCl and 5g Difco Yeast extract per liter), which in the case of LE392 was supplemented with 0.2% maltose and 10mM $MgSO_4$. λ phages were propagated using routine methods (21); the recombinant λ phages described in this paper were all isolated from an EMBL4 library, three of them by Baylis and Bibb (6) and the other three as described in this paper.

pBSCR8 contains a 7.5 kb BamHI insert harbouring the *rrnA* operon, pBSCR8-U a 5.5 kb EcoRI insert containing sequences upstream of *rrnA*.

* To whom correspondence should be addressed

+ Present address: John Innes Institute, Colney Lane, Norwich NR4 7UH, UK

DNA isolation and handling

Genomic DNA was isolated as described by Hopwood at al. (15). Small and large scale plasmid isolations were carried out according to an adaptation of the method of Birnboim and Doly (22). λ DNA was prepared according to Silhavy et al. (23). Cloning and subcloning were performed by standard procedures (21).

DNA sequencing

Subclones were made in M13mp18 and M13mp19 and sequenced using the T7 polymerase kit obtained from Pharmacia; the deaza sequencing kit (from the same supplier) was used for some GC rich parts. Both strands were sequenced at least once to minimise sequencing errors.

Computer analysis

Computer analysis of the sequence was performed using the UWGCG software (24). The programs 'Gap' and 'Codonpreference' were used for sequence alignments and for the prediction of open reading frames respectively. We used the most recent table available for codon usage in streptomyces (25).

Southern hybridisation

DNA samples were run in 0.7% agarose in TAE and blotted to Hybond N (Amersham), using 20×SSC as blotting buffer. Hybridisation conditions were: 16 hr at 65°C in 6×SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 1mM EDTA, 2.5% Denhardt solution and 100 μg/ml calf thymus DNA.

Filters were washed at the same temperature in 0.1% SDS and decreasing SSC concentrations until background signals were sufficiently low. DNA probes (10−50 ng) were labeled by random priming with hexanucleotides (26,27).

Bacteriophage λ plaque screening

Recombinant λ phages were plated at a density of approximately 6 plaques/cm^2 and duplicate replica filters were made by the method of Silhavy et al. (23). Hybridisation of the filters was performed as described above.

RESULTS

Cloning of the operons rrnA, rrnC and rrnE

The ribosomal RNA operons of *S.coelicolor* are organised in six gene sets, as was demonstrated recently by Baylis and Bibb (6). Further studies by these authors (14) led to the cloning of the *rrnB*, *rrnD* and *rrnF* operons. As a prelude to the cloning of the remaining three operons, we submitted chromosomal DNA of *S.coelicolor* A3(2) to a Southern analysis. After digestion of the DNA with the enzymes BamHI, BglII, EcoRI, HindIII, PstI or XhoI, fragments were separated by electrophoresis on a 0.7% agarose gel, blotted to nitrocellulose and hybridised with a 570 bp HindIII fragment of *E.coli rrnB* encompassing the nucleotides +80 to +650 of the 16S rRNA gene (11) (Figure 1^A). Since neither BamHI nor BglII nor XhoI recognise sites within the structural parts of the 16S rRNA genes, only one signal per gene is observed on the Southern blot upon use of these enzymes. The length of each fragment is known (6).

As is obvious from Figure 1^A, not all genes hybridise to the same extent with the HindIII fragment. On the basis of the intensities of the hybridisation signals at least two different operon groups can be discerned. This is in contrast to the outcome of a similar experiment by Baylis and Bibb (6) who observed six

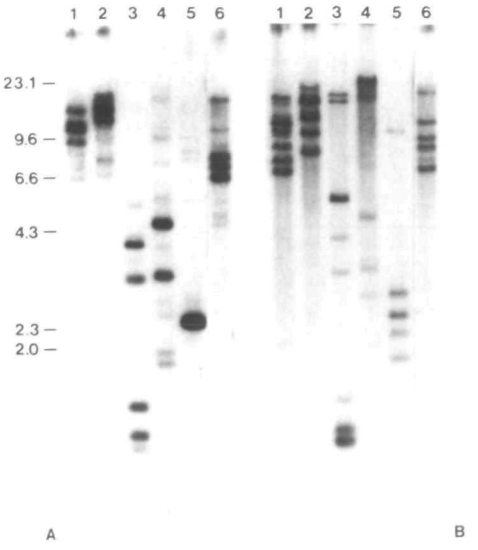

Fig. 1. Southern hybridisation of *S.coelicolor* chromosomal DNA with probes from *E.coli rrnB* and *S.coelicolor rrnA*. Digestions: 1. BamHI, 2. BglII, 3. EcoRI, 4. HindIII, 5. PstI, 6. XhoI. **1^A**:Hybridisation with the 570 bp HindIII fragment containing the +80/+650 segment of the *E.coli rrnB* 16S rRNA gene. **1^B**:Hybridisation with the 650 bp AccI-EcoRI fragment containing the −5/+645 segment of the *S.coelicolor rrnA* 16S rRNA gene. DNA fragment sizes are estimated on the basis of size markers derived from a HindIII digest of λ DNA, indicated on the left side of the figure.

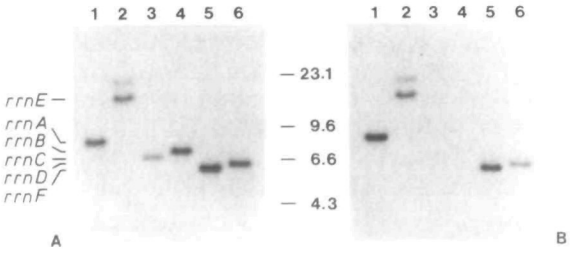

Fig. 2. Southern hybridisation of DNA of the recombinant λ clones containing the six operons of *S.coelicolor*. The DNA was digested with SalI prior to hybridisation. DNA size markers (in kb) and operon classifications are presented in the middle and on the left side of the picture, respectively. Lanes: 1.λSCR8, 2.λSCR18, 3.λSCR10, 4.λRSC28, 5.λRSC31, 6.λRSC33. **2^A**:Hybridisation with the 650 bp AccI-EcoRI probe containing the −5/+645 segment from the *S.coelicolor rrnA* 16S rRNA gene. **2^B**:Hybridisation with the 320 bp BglII probe containing the −600/−225 segment of the upstream region of *S.coelicolor rrnA*.

bands of approximately equal intensities, using labeled 5S, 16S and 23S rRNA from *S.coelicolor* as probes for hybridisation respectively. The possibility may thus be envisioned that *S.coelicolor* produces two classes of rRNA.

Cloning of the operons *rrnA*, *rrnC* and *rrnE* was performed starting with BamHI fragments, which are better resolved electrophoretically than the SalI fragments on which the operon nomenclature was based originally (6). *rrnB* corresponds to the 9 kb BamHI fragment, *rrnD* to the 12.8 kb BamHI fragment and *rrnF* to the 6.5 kb BamHI fragment. The nucleotide sequence of the 16S rRNA gene of *rrnD* and its flanking region is known (13,14). Considering the differences in hybridisation we chose to clone the 7.5 kb fragment (cf. Figure 1^A), which gives a weaker signal than the fragment corresponding to *rrnD*, the

operon best characterised so far. BamHI fragments of 7–9 kb were cloned in pBR329 and plasmid DNA was isolated from 600 colonies in pools of 24. The DNA was cut, submitted to agarose gel electrophoresis and blotted to nitrocellulose. Pools with the correct insert were identified on the basis of hybridisation signals observed with the 570 bp HindIII probe. After repeated colony purification and screening, two DNA minipreparations yielding an unambiguous positive signal were obtained. BamHI digestion proved both to contain a 7.5 kb insert. The clones, further shown to be identical by means of restriction mapping, were designated pBSCR8. They were shown to contain the *rrnA* operon, as is demonstrated below.

To clone the *rrnC* and *rrnE* operons we screened an EMBL 4 library containing *S.coelicolor* A3(2) M145 DNA (prepared by Dr. J.S. Feitelson, John Innes Institute, Norwich, U.K.). Positive recombinant λ phages were identified by hybridising with a 650 bp AccI-EcoRI fragment containing the 5′ half of the 16S rRNA gene of *rrnA* (cf. Figure 3). Screening of approximately 800 plaques yielded 13 positive signals. The corresponding plaques were purified, recombinant λ DNA was isolated and hybridised with the same probe. In this way seven λ clones, yielding an unambiguous positive signal, were obtained. For further characterisation, recombinant λ DNA was digested with BamHI. After Southern hybridisation with the AccI-EcoRI probe, four clones appeared to contain a 7.5 kb BamHI fragment corresponding to pBSCR8. These were designated λSCR8. Two clones were shown to correspond to the 10.5 kb fragment and were designated λSCR10, while one clone, corresponding to a 18 kb BamHI fragment was designated λSCR18.

Identification of the *rrn* operons carried by the recombinant λ phages was performed by Southern analysis of the phage DNA after digestion with SalI (this article and (6)) and probing with the AccI-EcoRI fragment from pBSCR8. The results confirmed that λSCR8 and λSCR10 contained *rrnA* and *rrnC*, respectively, (Figure 2^A). λSCR18 yields a hybridisation signal corresponding to a SalI fragment of more than 18 kb, which must have arisen from the loss of a SalI site either upstream or downstream of the operon, since previous experiments had shown that the *rrn* operons were contained in SalI fragments no larger than 9 kb (this article and (6)). The restriction map of λSCR18 shows however, that the operon carried by this phage differs from the five other *rrn* operons and therefore should be *rrnE* (data not shown). The results are summarised in Table 1.

Table 1 Classification of the six rRNA operons of *S.coelicolor* on the basis of the length of the restriction fragments (in kb) containing the rRNA operons, as shown by hybridisation (this article, Figure 1 and reference 6).

BamHI	SalI	Operon	Clone	Reference
18	6.5	*rrnE*	λSCR18	this paper
12.8	6.5	*rrnD*	λRSC33	6
10.5	7.0	*rrnC*	λRSC10	this paper
9.0	7.5	*rrnB*	λRSC28	6
7.5	8.7	*rrnA*	λSCR8	this paper
6.5	5.7	*rrnF*	λRSC31	6

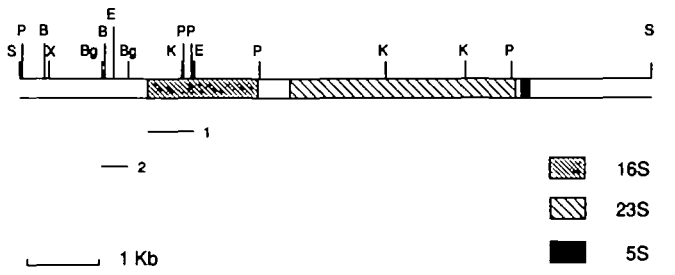

Fig.3. Restriction map of *S.coelicolor rrnA* and the upstream region. Probes derived from this operon used in hybridisation studies are shown below the map. **Abbreviations of restriction enzymes:** B = BamHI, Bg = BglII, E = EcoRI, K = KpnI, P = PstI, S = SalI, X = XhoI. **Probes:** 1 = 650 bp AccI–EcoRI; 2 = 320 bp BglII.

Sequence differences upstream of the rrn promoters

As mentioned above, a Southern restriction analysis of *S.coelicolor* M145 DNA using the 570 bp HindIII fragment of *E.coli rrnB* that is internal to the native 16S rRNA as a probe shows pronounced differences in hybridisation signals for the six operons. For instance, the BamHI fragments of 6.5 kb, 7.5 and 9 kb in lane 1 of Figure 1^A corresponding to *rrnF*, *rrnA* and *rrnB* respectively, hybridise much weaker than the 10.5 kb, 12.8 kb and 18 kb fragments corresponding to *rrnC*, *rrnD* and *rrnE* respectively. That these signal differences are not due to experimental artifacts can be concluded from the experiment shown in Figure 1^B, in which the same blot, after removal of the HindIII probe, was hybridised with the 650 AccI-EcoRI fragment containing the 5′ half of the 16S rRNA gene of *rrnA*. Now the BamHI fragments derived from *rrnA*, *rrnB* and *rrnF* yield strong signals, comparable to those of *rrnC*, *rrnD* and *rrnE*. Why probing with two different probes, one derived from *E.coli rrnB* the other from *S.coelicolor rrnA* but both encompassing sequences from the 5′ half of the 16S rRNA gene, leads to such a different result remains to be explained (see also the Discussion). It cannot be ascribed to a certain amount of partial digestion observable in Figure 1: the smaller BamHI fragments of 6.5 kb and 7.5 kb of Figure 1^B clearly show a very strong hybridisation in contrast to those of Figure 1^A.

High variation in hybridisation intensities is seen when SalI digested DNA of the six recombinant λ clones is submitted to Southern restriction analysis, using a 320 bp BglII fragment containing part of the upstream region of *rrnA* (cf. Figure 3) for probing. As can be seen in Figure 2^B the *rrnA* and *rrnF* bands display approximately equal hybridisation, whereas the *rrnB* and *rrnC* bands are hardly visible. These data are best explained by assuming that the 16S rRNA genes of the operons are very homologous, but that *rrnA* and *rrnF* differ from the *rrnB* and *rrnC* in their upstream regions between positions −600 and −250 (in respect to the start of the 16S rRNA gene).

Nucleotide sequence of rrnA

The apparent inconsistency in hybridisation with two different probes prompted nucleotide sequence analysis of the 16S rRNA gene of *rrnA* and its upstream region. Figure 3 shows the restriction map of the 8.7 kb SalI fragment from λSCR8 containing *rrnA*. Figure 4^A shows the nucleotide sequence of the 16S rRNA gene. In Figure 4^B the nucleotide sequence of the

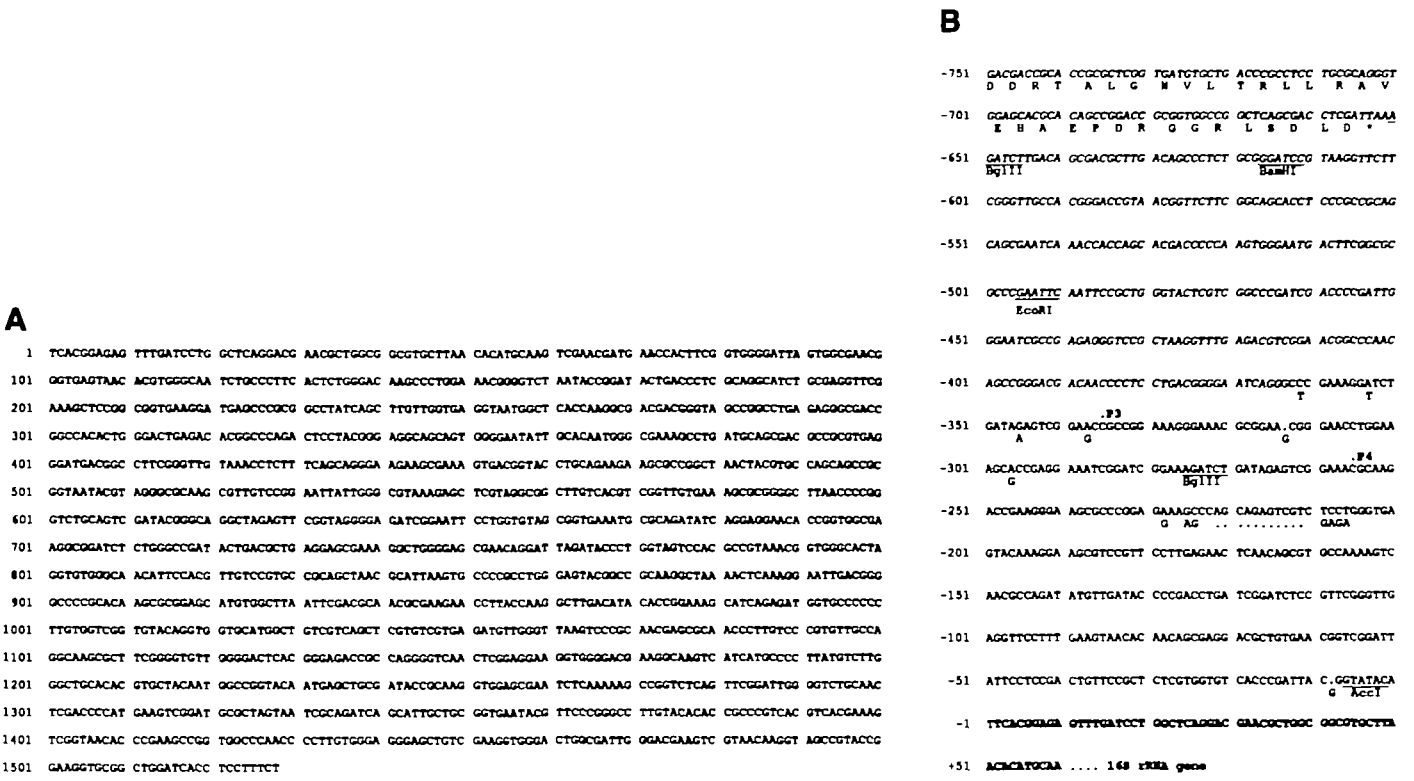

Fig.4. Nucleotide sequence of the *S.coelicolor rrnA* 16S rRNA gene and upstream region. 4^{A}:Nucleotide sequence of the 16S rRNA gene. 4^{B}:Comparison of the nucleotide sequence of the upstream region of *rrnA* with the corresponding region of *rrnD*. Dots indicate a gap in the sequence, sequences in italics differ so strongly from *rrnD* that no reasonable comparison could be made. Some important restriction sites are presented by underlining and name. Putative transcription start sites (by sequence homology with the P3 and P4 promoter of *S.coelicolor rrnD*) are indicated with a dot above the sequence. Amino acid translation of the putative ORF is shown below the sequence.

upstream region and the start of the 16S rRNA gene are compared to the corresponding region of *rrnD*. The identity of the 16S rRNA genes of *rrnA* and *rrnD* is 99%, the only relatively variable region being located around +600. A putative open reading frame was detected by computer analysis, ending at −653. The implications of the sequence analysis are discussed below.

DISCUSSION

With the cloning of the rRNA operons *rrnA*, *rrnC* and *rrnE* of *S.coelicolor* reported in the present paper, the cloning of all six *rrn* operons of this microorganism is now complete (see also (6)). Southern hybridisation analysis performed with a probe derived from *E.coli* suggested a structural difference between the 16S rRNA genes of at least some of these operons. Since the homology between rRNA genes of all microorganisms studied so far, for example *B.subtilis* (28), *E.coli* (5) and *Rhodobacter sphaeroides* (29), is more than 95%, this was not expected. Nucleotide sequence analysis of the 16S rRNA gene of *rrnA* revealed, however, a 99% homology with the corresponding *rrnD* gene. On the other hand differences are found in the upstream regions. They are particularly pronounced between positions −750 and −364 relative to the starts of the *rrnA* and *rrnD* 16S rRNA genes (positions 1 to 387 of Figure 4^{B}, respectively). Previously, the *rrnD* promoters P1 and P2 have been localised in this region (14). The lack of any relevant sequence similarity makes the presence of promoters in this region unlikely. However, there is no true consensus for *Streptomyces* promoters (30), which renders promoter identification based on sequence comparison perilous. Moreover, P1 and P2 contribute only weakly to the transcription of *rrnD* in vegetative mycelium (Bibb and Clayton, personal communication).

A putative open reading frame is found, ending at −650 relative to the start of the 16S rRNA gene of *rrnA*. The corresponding amino acid sequence is shown below the DNA sequence. This ORF differs from the ORF located upstream of *rrnD* in sequence and position relative to the 16S rRNA gene start. What the function of these ORFs is remains to be seen.

Putative transcription start sites of *rrnA* are indicated in Figure 4^{B} and are based on sequence similarities displayed by the P3 and P4 promoters of *rrnD*. Further studies are needed to establish that transcription initiation does occur at these sites (as has been done in the case of the *rrnD* operon).

The differences revealed by the sequence analysis of the 16S rRNA gene upstream region, particularly that upstream of P3 and P4, are intriguing. If regulatory sequences are found there, the possibility exists that transcription of *rrnA* and *rrnD* is differentially controlled. The question also arises to which extent the other four *rrn* operons differ in this respect. The Southern hybridisation of SalI-digested recombinant λ DNA derived from the six operons is highly intriguing. Restriction fragments of *rrnB* and *rrnC* do not hybridise (or at a very low level) with a probe encompassing the upstream −650 to −225 region of *rrnA*. Fragments of *rrnD* hybridise much weaker than fragments of *rrnA*

and *rrnF*. These results suggest that the upstream regions of some of the *rrn* operons vary significantly, urging further structural and functional analyses which are in progress in this laboratory.

As mentioned above the 16S rRNA genes of *rrnA* and *rrnD* display a very strong homology (99%). However, small differences are found downstream of the start of the 16S rRNA gene around position +600. The homology between this region of *rrnA* is stronger with the corresponding region of *S.ambofaciens rrnD* than with that of *S.coelicolor rrnD*. If sequencing errors can be excluded, this is surprising from a phylogenetic point of view.

Finally, the small difference in 16S rRNA sequence of *rrnA* and *rrnD* does not explain the difference in hybridisation observed with 16S rRNA probes of different origin used in the Southern analyses shown in Figures 1A and 1B. Apparently, in the case of a heterologous probe hybridisation strength is influenced by sequences upstream of the 16S rRNA gene start, although the region that actually hybridises is located downstream of this start. This result calls for caution in interpreting hybridisation data of this type and emphasises the necessity of direct sequence determination.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. Mervyn Bibb for carefully reading the manuscript and fruitful discussions, Dr. Tim Clayton for providing us with the recombinant λ clones λSCR28, λSCR31 and λSCR33 and Dr. Keith Chater for sending the EMBL 4 library of *S.coelicolor* A3(2) chromosomal DNA.

REFERENCES

1. Enquist,L.W. and Bradley,S.G. (1971) Devel. Ind. Microbiol. 12, 225–236.
2. Chater,K. (1984) in Losick,R. and Shapiro,L. (eds.), Microbial development, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 89–116.
3. Bérdy,J. (1980) Proc. Biochem. Oct/Nov, 28–35.
4. Nomura,M., Gourse,R. and Baughman,G. (1984) Ann. Rev. Biochem. 158, 165–170.
5. Lindahl,L. and Zengel,J.M. (1986) Ann. Rev. Genet. 20, 7–326.
6. Baylis,H.A. and Bibb,M.J. (1988) Mol. Gen. Genet. 211, 191–196.
7. Suzuki,Y., Ono,Y., Nagata,A. and Yamada,T. (1988) J. Bacteriol. 170, 1631–1636.
8. Kim,E., Kim,H., Kang,K.H., Kho,Y.H. and Park,Y-H. (1991) Nucl.Acids Res. 19, 1149.
9. Plohl,M. and Gamulin,V. (1991) FEMS Microbiol. Lett. 77, 139–144.
10. Pernodet,J.-L., Boccard,F., M.T. Alegre, Gagnat,J. and Guérineau,M. (1989) Gene 79, 33–46.
11. Brosius,J., Dull,T.J., Sleeter,D.D. and Noller,H.F. (1981) J. Mol. Biol. 148, 107–127.
12. Suzuki,Y., Nagata,A., Ono,Y and Yamada,T. (1988) J. Bacteriol. 170, 2886–2889.
13. Baylis,H.A. and Bibb,M.J. (1987) Nucl. Acids Res. 15, 7176.
14. Baylis,H.A. and Bibb,M.J. (1988) Mol. Microbiol. 2, 569–579.
15. 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, U.K.
16. Covarrubias,L. and Bolivar,F. (1982) Gene 17, 79–89.
17. Norrander,J., Kempe,T. and Messing,J. (1983) Gene 26, 101–106.
18. Messing,J., Crea,R. and Seeburg,P.H. (1981) Nucl. Acids Res. 9, 309–321.
19. Yanish-Perron,C., Vieira,J. and Messing,J. (1985) Gene 33, 103–119.
20. Murray,N.E., Brammer,W.J. and Murray,K. (1977) Mol. Gen. Genet. 150, 53–61.
21. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, Second Edition.
22. Birnboim,H.C. and Doly,J. (1979), Nucl. Acids Res. 7, 1513–1523.
23. Silhavy,T.J., Berman,M.L. and Enquist,L.W. (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
24. Devereux,J., Haeberli,P. and Smithies,O. (1984) Nucl. Acids Res. 12, 387–395.
25. Hopwood,D.A. and Kieser,T. (1990) in Drlica,K. and Riley,M. (eds) The bacterial Chromosome, American Society for Microbiology, Washington DC, pp. 147–162.
26. Feinberg,A.P. and Vogelstein,B. (1983) Anal. Biochem. 132, 6–13.
27. Feinberg,A.P. and Vogelstein,B. (1984) Anal. Biochem. 137, 266–267.
28. Loughney,K., Lund,E. and Dahlberg,J.E. (1982) Nucl. Acids Res. 10, 1607–1624.
29. Dryden,S.C. and Kaplan,S. (1990) Nucl. Acids Res. 18, 7267–7277.
30. Hopwood,D.A., Bibb,M.J., Chater,K.F., Janssen,G.R., Malpartida,F. and Smith,C. (1986) in Booth,I. and Higgins,C. (eds.), symposium of the Society for General Micriobiology. Regulation of gene expression. Cambridge university press. pp. 251–276.