Mol Gen Genet (1986) 202:416–420

MGG

The copy number of *Bacillus* plasmid pRBH1 is negatively controlled by RepB protein

Takashi Ano, Tadayuki Imanaka, and Shuichi Aiba
Department of Fermentation Technology, Faculty of Engineering, Osaka University, Yamada-oka, Suita-shi, Osaka 565, Japan

Summary. The replication control mechanism of *Bacillus* plasmid pRBH1 was analysed; pRBH1 contains four promoters, P1 to P4, and a large inverted repeat (63 base pairs) upstream of the protein (RepB) coding sequence. The stem and loop structure is surrounded by two promoters, P1 and P3, with different directions of transcription. One base substitution in the loop structure caused a change in copy number. Since the P1 promoter is located upstream of the replication origin of pRBH1, the transcript from the P1 promoter might serve as the primer of DNA replication. In vivo transcription from the P1 promoter was repressed by a *trans*-acting plasmid gene product. Since the RepB protein is involved in copy number control and RepB contains the consensus amino acid sequence of DNA binding proteins, RepB was thought to be the repressor. It was concluded from these data that the inverted repeat is involved in the control of copy number of the plasmid pRBH1. The RepB protein also contains two regions highly homologous with the Rom protein encoded on *Escherichia coli* plasmid ColE1. The possible mechanism for the copy number control of the plasmid via RepB protein and/or RNAs is discussed.

Key words: Thermophilic *Bacillus* plasmid pRBH1 – Copy number control – Homology between RepB and Rom proteins – Inverted repeat – Promoter activities in vivo

Introduction

Studies on plasmid replication have been focused on the control mechanism, and significant progress has been made for many *Escherichia coli* plasmids (Scott 1984). However, the replication control of *Bacillus* plasmids has not yet been intensively studied. Plasmids pTB913 and pUB110 replicate in both *Bacillus subtilis* and *Bacillus stearothermophilus* (Imanaka et al. 1982, 1984). The nucleotide sequences of essential replication origin regions of these plasmids have been found to be identical to each other except for one base (Muller et al. 1986). According to previous work, the replication origin and flanking regions include four promoters, a Shine-Dalgarno sequence, an inverted repeat and a protein coding sequence as shown in Fig. 1. The protein product, RepB, is involved in the copy number control of these plasmids as shown by the study on mutant plasmids with increased copy numbers.

Offprint requests to: S. Aiba

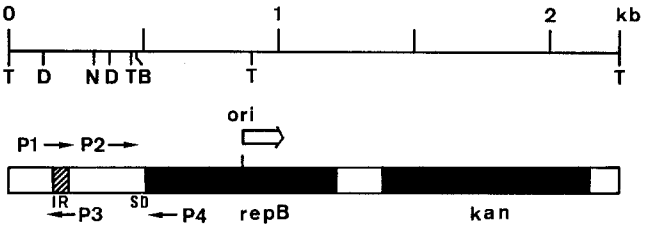

Fig. 1. Genetic structure of plasmid pRBH1. *Closed bars* indicate protein-coding regions of *repB* and *kan*. The *hatched region* indicates an inverted repeat (IR). P and SD denote RNA polymerase and ribosome binding sites, respectively. *Arrows*, → and ⇒, indicate the directions of transcription and replication, respectively. Restriction endonuclease cleavage sites are drawn below the scale line: T, *Taq*I; D, *Dra*I; N, *Nco*I; B, *Bst*NI

The purpose of this paper is to demonstrate that the inverted repeat is one of the plasmid copy number control regions and that plasmid replication is negatively regulated by a *trans*-acting gene product such as the RepB protein. The mechanism of plasmid copy number control in *Bacillus* is discussed by analogy with that of plasmid ColE1 in *E. coli*.

Materials and methods

Bacteria and plasmids. The bacterial strain and plasmids used are listed in Table 1; *B. subtilis* MI113 was used as the host cell for the plasmids. The copy number mutant plasmids pRBHC3 and pRBHC7 had only single base substitutions, at different positions in the RepB coding region of pRBH1 (Muller et al. 1986). Plasmid pPF001, used as a promoter probe vector, consisted of the penicillinase structural gene and pHV14.

Preparation of plasmid DNA. Plasmid DNA was prepared either by the alkaline phenol procedure or the cleared lysate method, followed by CsCl-ethidium bromide equilibrium density gradient centrifugation as described previously (Matsumura et al. 1984; Imanaka et al. 1985).

Transformation. Transformation of *B. subtilis* competent cells was performed as described previously (Imanaka et al. 1981a). Transformants were selected on L-agar plus kanamycin (Km, 5 μg/ml) or chloramphenicol (Cm, 30 μg/ml).

Table 1. Bacterial strain and plasmids

Strain		Characteristics	Reference
Bacillus subtilis MI113		*arg-15 trpC2* $r_M^- m_M^-$	Imanaka et al. (1981a)
Plasmid	**Molecular size (bp)**	**Characteristics**	**Reference**
pUB110dB	2,266	Km^r	Muller et al. (1986)
pRBH1	2,262	Km^r	Muller et al. (1986)
pRBH1·IR	2,262	Km^r	This work
pRBHC3	2,262	Km^r	Muller et al. (1986)
pRBHC3·IR	2,262	Km^r	This work
pRBHC7	2,262	Km^r	Muller et al. (1986)
pRBHC7·IR	2,262	Km^r	This work
pHV14	7,273	Cm^r	Imanaka et al. (1982)
pPF001		Cm^r	This work
pPF301		Cm^r	This work

Cleavage of DNA with restriction enzymes, repair of cohesive ends and ligation of DNA. Treatment of DNA with restriction enzymes (*Dra*I, *Hha*I, *Nco*I, *Taq*I, *Bst*NI), repair of cohesive ends with the Klenow fragment of DNA polymerase I and ligation of DNA with T4 DNA ligase were done following the protocols of the manufacturers. The enzymes used in these experiments were purchased from commercial suppliers.

Gel electrophoresis for DNA isolation. Agarose gel electrophoresis and DNA isolation from low melting point (LMP) agarose gels have been described elsewhere (Imanaka et al. 1985).

Assessment of plasmid copy number. The plasmid copy number was assessed as described by Muller et al. (1986).

Detection of penicillinase-positive colonies on plates and penicillinase assay. Penicillinase was assayed by the iodometric method as described previously (Imanaka et al. 1981b). Detection of penicillinase-positive colonies on plates has been described earlier (Imanaka et al. 1981b).

Results

Copy number change caused by one base substitution in the loop structure

Nucleotide sequence analyses of *Bacillus* plasmids pRBH1 (the smallest derivative of pTB913; Muller et al. 1986) and pUB110dB (the smallest derivative of pUB110; Muller et al. 1986) disclosed the presence of a large inverted repeat upstream of the protein (RepB) coding sequence (Muller et al. 1986). A possible secondary structure of this inverted repeat is shown in Fig. 2. The stem and loop structure of pRBH1 consists of 63 bases and 46 bases are paired. At position −316 there is a single base substitution (C → T) in pUB110dB, besides the slight changes of nucleotide sequences upstream of the loop. The copy number of

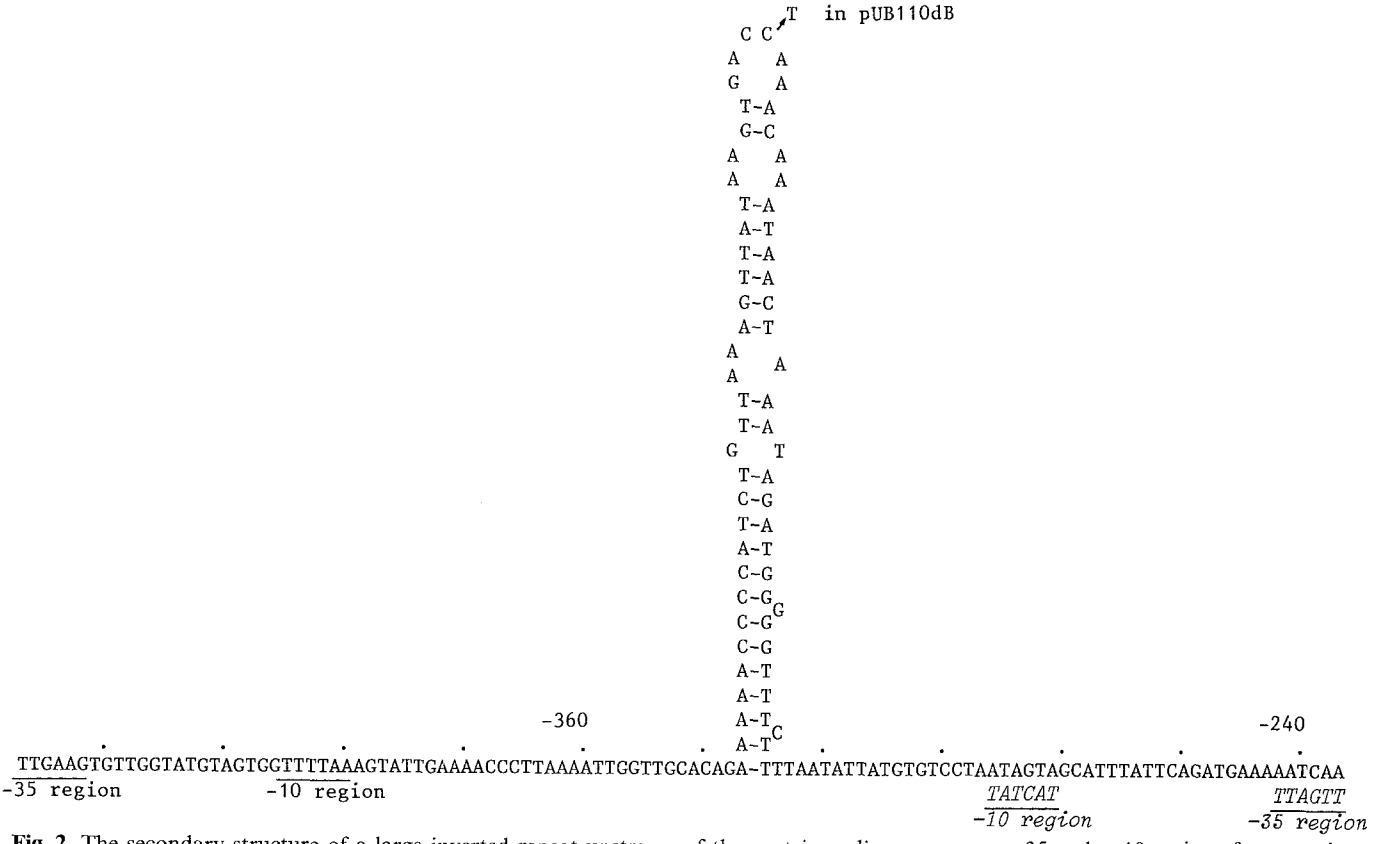

Fig. 2. The secondary structure of a large inverted repeat upstream of the protein coding sequence. −35 and −10 regions for opposite transcriptional directions are shown in *italics*. The one nucleotide difference in pUB110dB and pRBH1 is indicated by an *arrow*. The position of the first nucleotide of the RepB protein coding region is defined as +1 (Muller et al. 1986)

Table 2. Copy number of one base substituted plasmids

Plasmid	Copy number[a]
pRBH1	42
pRBH1·IR	134
pRBHC3	134
pRBHC3·IR	214
pRBHC7	202
pRBHC7·IR	214

[a] Copy number was assessed at 37° C

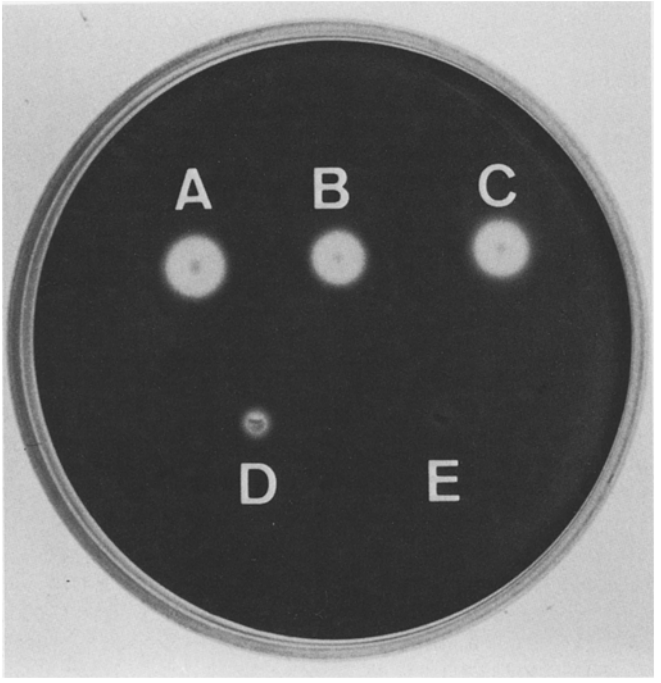

Fig. 3. Assay for penicillinase activity on polyvinyl alcohol plates. Cells were incubated at 37° C for about 7 h. Penicillinase-positive colonies A, B, and C show the existence of promoters functioning in vivo. Plasmids carried by *Bacillus subtilis* are as follows: A, pPF001-P1; B, pPF001-P2; C, pPF001-P4; D, pPF001; E, pHV14

pUB110dB is larger than that of pRBH1 (Muller et al. 1986), hence the substitution in the loop structure might have caused the difference in copy number between pUB110dB and pRBH1.

To simplify the picture, only the base substitution (C→T) was introduced into the loop structure of the inverted repeat of pRBH1 and also into that of its copy number mutant plasmids pRBHC3 and pRBHC7. This was done as follows: pUB110dB was digested with *Dra*I, and a 246 bp *Dra*I fragment which contained the large inverted repeat (IR; Fig. 1) was isolated from an LMP agarose gel. The *Dra*I fragment (246 bp) was ligated with the larger *Dra*I fragment of pRBH1 and the recombinant plasmid was designated as pRBH1·IR. Likewise, pRBHC3·IR and pRBHC7·IR were obtained from pRBHC3 and pRBHC7, respectively. The copy numbers of these plasmids, pRBH1·IR, pRBHC3·IR and pRBHC7·IR, were larger than those of the original plasmids pRBH1, pRBHC3 and pRBHC7 (Table 2). These results show that this inverted repeat is related to the copy number control of plasmids.

Table 3. Effect of coexisting plasmid on the activities of the *penP* gene directed by the P1 promoter

Plasmids	Penicillinase activity		Relative copy number of pPF301-P1
	Units/OD_{660}	Ratio (%)	
pPF301-P1	5,600	100	1
pPF301-P1 + pRBH1	87	2	0.5
pPF301-P1 + pRBH1·IR	200	4	0.8
pPF301-P1 + pRBHC3	330	6	0.5
pPF301-P1 + pRBHC3·IR	290	5	0.6
pPF301-P1 + pRBHC7	310	6	0.5
pPF301-P1 + pRBHC7·IR	370	7	0.5

Detection of promoter activities in vivo

Promoter activities in vivo were examined by using the promoter probe vector pPF001. Three fragments, i.e. *Taq*I-*Nco*I (containing promoters P1 and P3), *Nco*I-*Taq*I (containing promoter P2), and *Taq*I-*Taq*I (containing promoter P4) were isolated from pRBH1 (Fig. 1). Each fragment was repaired with the Klenow fragment of DNA polymerase I and ligated with a linearized pPF001 fragment which had been digested with *Bam*HI and repaired. These ligation mixtures were used to transform *B. subtilis* and Cm^r transformants were assayed for penicillinase on plates.

Penicillinase-positive colonies were found with each ligation mixture as shown in Fig. 3. These results indicate that promoters P1 and/or P3, P2, and P4 function in *B. subtilis*. Plasmids containing P1, P2 or P4 were designated as pPF001-P1, pPF001-P2 or pPF001-P4, respectively. Plasmids containing the *Taq*I-*Nco*I fragment (with P1 and P3) were isolated, and the orientation of the fragment was analysed. The same direction exhibiting P1 activity was found for all the plasmids tested, and the reverse direction for P3 activity was not found at all. Since the DNA sequences of promoters P1 to P4 are similar (Muller et al. 1986), it is most plausible to assume that the promoter P3 might also have functioned in vivo. However, the apparent absence of P3 activity could be explained by the transcriptional termination that will be referred to later on (see Discussion).

Inhibition of in vivo transcription from promoter P1

Since promoter P1 is located upstream of the replication origin of pRBH1, a transcript from the P1 promoter might serve as the primer of DNA replication. The possibility that transcription from the P1 promoter might be affected by a *trans*-acting plasmid gene product was examined as follows: pBR322 was removed from pPF001-P1 to minimize the influence of pBR322 on the assay of P1 promoter activity. The plasmid thus obtained was designated pPF301-P1.

Transformation of *B. subtilis* carrying resident plasmid pPF301-P1 was carried out with either pRBH1 or its derivatives. Transformants were cultivated in L-broth plus Cm and Km and penicillinase activities were assayed (Table 3). The synthesis of the enzyme was strongly repressed by the presence of pRBH1 or any one of its derivatives. Although the copy number of pPF301-P1 was slightly reduced (to about one-half at most) in the presence of the various plas-

mids, the enzyme activity was remarkably repressed (Table 3). This means that the marked repression was not caused mainly by the decrease in the copy number of pPF301-P1. Accordingly, transcription from the P1 promoter was apparently repressed by a *trans*-acting plasmid gene product, the RepB protein, encoded in the replication region (Fig. 1; Muller et al. 1986).

Discussion

The plasmid pRBH1, having the same DNA sequence in the replication region as pUB110 except for one base, contains both the gene for RepB protein and the replication origin (Muller et al. 1986). Mutations in plasmid pRBH1, designated C3 or C7, resulted in an elevated plasmid copy number in *B. subtilis* (Muller et al. 1986; Table 2). The nucleotide sequence analysis in the previous paper (Muller et al. 1986) has shown that these mutations are single amino acid substitutions (Gly by Asp for C3, and Gly by Glu for C7). In this work also, one base substitution in the large inverted repeat was shown to cause a change in copy number (Table 2). This inverted repeat is surrounded by the two counter promoters, P1 and P3. Promoter P1 functioned in *B. subtilis* (Fig. 3) and transcription from P1 was repressed by the *trans*-acting gene product (RepB; Table 3). Plasmid pRBH1 has unique sites for *Nco*I and *Bst*NI between the P1 promoter and the RepB protein coding region (Fig. 1). When pRBH1 was treated with either *Nco*I or *Bst*NI, and repaired with the Klenow fragment of DNA polymerase I, no transformants of *B. subtilis* were obtained from either ligation mixture (data not shown). These facts suggest that RNA1 (transcript from P1) might be important in situ in plasmid DNA replication.

Assuming that RNA1 serves as a primer for the replication of pRBH1, the repression of transcription from the P1 promoter (Table 3) would imply the presence of negative control by RepB in the initiation of DNA replication. Similar observations have been reported on ColE1 by two groups. Transcription from the RNA primer promoter was repressed by the *trans*-acting plasmid gene product (Rom, 63 amino acid protein), using the *galK* gene (Som and Tomizawa 1983). Cesareni et al. (1982) have also reported that in vivo expression of the *lacZ* gene, whose transcription was directed by the primer promoter of ColE1, was repressed by a 63 amino acid protein and speculated that the protein was a repressor of synthesis of the primer.

If RepB functions as a repressor, the protein should be bound to DNA. Therefore, we searched in the RepB protein (Muller et al. 1986) for a specific amino acid sequence similar to the consensus sequence [*Ala*-N-N-N(hydrophobic)-*Gly*-N(hydrophobic)-N-N-N-N-*Val*(*Ile*)] found in many DNA-binding proteins (Pabo and Sauer 1984). In fact, such an amino acid sequence was found [Thr-Val-Glu-Thr-*Ala*-Arg-Met-Phe-*Gly*-Val-Leu-Asn-Lys-Asn-*Ile*-Lys-Lys-Tyr-Gln-Met(73-54)], the direction being from carboxyl to amino terminus (Muller et al. 1986). This finding accords with the argument that the RepB protein functions as a repressor.

According to Tomizawa (1984), a region 400–555 bp upstream from the origin of DNA replication is involved in the control of the copy number of ColE1. This particular region contains palindromes and codes for a part of the primer precursor on one strand and on the other strand, for a small RNA. Initiation of ColE1 DNA replication is inhibited by the small RNA by interaction with the complementary structure in the primer precursor, and the inhibitory action of RNAI is increased by the Rom protein which enhances binding of complementary RNAs (Tomizawa and Som 1984).

The copy number control region of pRBH1 also has a large inverted repeat surrounded by two counter promoters. The structural resemblance of pRBH1 and ColE1 suggested a similar control mechanism by RNA molecules of plasmid DNA replication. Since the structure and function of the Rom protein has been studied intensively, we compared the amino acid sequence of the RepB protein with that of Rom. Two highly homologous regions were found in the RepB protein (Fig. 4). One region at the amino terminal sequence (7–65) of RepB exhibited about 30% homology, and the other at the carboxyl terminal sequence (168–231) showed 41% homology. This homology indicates that the RepB protein behaves like the Rom protein, of which the function is to enhance the binding of two complementary RNAs.

In summary, the following speculation on the copy number control mechanism has been presented (Fig. 5). The synthesis of RNA1 as a primer for the replication of the plasmid is repressed at IR by RepB as a negative control factor. In addition, the complementary small RNA molecule RNA3 interacts with RNA1 via IR, thus inhibiting DNA replication, as documented for ColE1 (Tomizawa 1984). This inhibitory effect of RNA3 on primer formation

```
RepB   SQKVVAEVIKQKPTVRWLFLTLTVK-NVYDGEE---LNKSLSDMAQGFRRMMQYKKINKNLVG  (7 - 65)
         =     *     *=   ===  = =  = *=   *  == = =    =    *
Rom    MTKQEKTALNMARFIRSQTLTLLEKLNELDADEQADICESLHDHADELYRSCLARFGDDGQNL  (1 - 63)

RepB   LSDLEE-GLHRKRLISYGGLLKEIHKKLNLDDTEEG-DLIHTDDDEKADEDGFSIIAMWNWERKNY  (168 - 231)
       **  =   =  = =    =   *  ===  = *=  =*  *  =  ===   = *= *  *  =
Rom    MTKQEKTALNMARFIRSQTLT--LLEKLNELDADEQADICESLHDH-ADELYRSCLARFGDDGQNL  (1 - 63)
```

Fig. 4. Comparison of the amino acid sequences of the RepB and Rom proteins. Identical amino acid residues are marked (=); conservative replacements are marked (*). Conservative replacements are defined as being within the groups: (D, E), (K, R), (S, T), (F, Y, W), and (I, L, V, M). Gaps have been inserted to improve alignment and are marked by *hyphens* within the sequences. The one-letter amino acid codes are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr

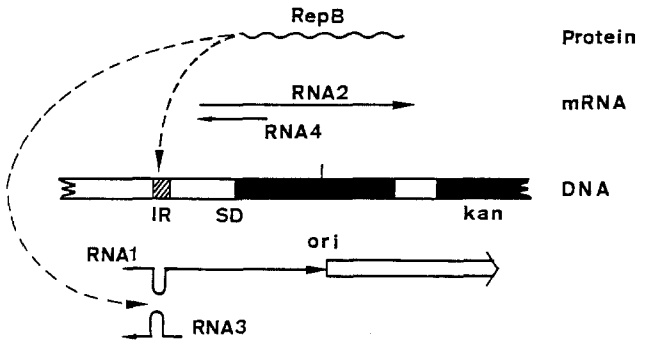

Fig. 5. A speculation on pRBH1 DNA replication control shown schematically. Transcripts from promoters P1 to P4 are designated as RNA1 to RNA4, respectively. RNA1, the primer of DNA replication, is synthesized from a region upstream of the origin but the small RNA3, which is transcribed in the opposite direction to RNA1, inhibits the formation of the primer. RepB protein encoded at the replication origin region is translated from RNA2. However, the synthesis of the protein is regulated by the small RNA4 which is complementary to RNA2. The RepB protein might interact with the inverted repeat (IR) region as a repressor, and enhance the binding of RNA1 and RNA3 by its Rom-like function

is enhanced by RepB in a similar fashion to that of the Rom protein of ColE1 (Tomizawa and Som 1984) because of the existence of an homologous amino acid sequence in the two proteins.

The synthesis of the RepB protein would be regulated at the translational level by masking the Shine-Dalgarno sequence of RNA2 with the small complementary RNA4. This regulation would contribute to the concentration of RepB protein in vivo at an appropriate level. Such regulation via the interference of ribosome binding to mRNA has already been studied for *ompC* in *E. coli* (Mizuno et al. 1984) and for *repA* in the R1 plasmid (Light and Molin 1983). Two small RNA species, of 80 and 250 nucleotides, have been detected in in vitro transcription experiments using pUB110 and the RNA polymerases of *E. coli* and *B. subtilis* (Brückner et al. 1984). The smaller one (80 nucleotides long) might correspond to RNA3 and the longer one to RNA4. The fact that P3 activity could not be detected in the promoter probe vector could be explained by RNA3 being too small to reach the penicillinase gene.

Acknowledgement. The authors would like to thank R.E. Muller for valuable discussions.

References

Brückner R, Zyprian E, Matzura H (1984) Expression of a chloramphenicol resistance determinant carried on hybrid plasmids in gram-positive and gram-negative bacteria. Gene 32:151–160

Cesareni G, Muesing MA, Polisky B (1982) Control of ColE1 DNA replication: The *rop* gene product negatively affects transcription from the replication primer promoter. Proc Natl Acad Sci USA 79:6313–6317

Imanaka T, Fujii M, Aiba S (1981a) Isolation and characterization of antibiotic resistance plasmids from thermophilic bacilli and construction of deletion plasmids. J Bacteriol 146:1091–1097

Imanaka T, Tanaka T, Tsunekawa H, Aiba S (1981b) Cloning of the genes for penicillinase, *penP* and *penI*, of *Bacillus licheniformis* in some vector plasmids and their expression in *Escherichia coli, Bacillus subtilis,* and *Bacillus licheniformis.* J Bacteriol 147:776–786

Imanaka T, Fujii M, Aramori I, Aiba S (1982) Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis.* J Bacteriol 149:824–830

Imanaka T, Ano T, Fujii M, Aiba S (1984) Two replication determinants of an antibiotic-resistance plasmid, pTB19, from a thermophilic bacillus. J Gen Microbiol 130:1399–1408

Imanaka T, Himeno T, Aiba S (1985) Effect of *in vitro* DNA rearrangement in the NH_2-terminal region of the penicillinase gene from *Bacillus licheniformis* on the mode of expression in *Bacillus subtilis.* J Gen Microbiol 131:1753–1763

Light J, Molin S (1983) Post-transcriptional control of expression of the *repA* gene of plasmid R1 mediated by a small RNA molecule. EMBO J 2:93–98

Matsumura M, Katakura Y, Imanaka T, Aiba S (1984) Enzymatic and nucleotide sequence studies of a kanamycin-inactivating enzyme encoded by a plasmid from thermophilic bacilli in comparison with that encoded by plasmid pUB110. J Bacteriol 160:413–420

Mizuno T, Chou M-Y, Inouye M (1984) A unique mechanism regulating gene expression: Translational inhibition by a complementary RNA transcript (micRNA). Proc Natl Acad Sci USA 81:1966–1970

Muller RE, Ano T, Imanaka T, Aiba S (1986) Complete nucleotide sequences of *Bacillus* plasmids pUB110dB, pRBH1 and its copy mutants. Mol Gen Genet 202:169–171

Pabo CO, Sauer RT (1984) Protein-DNA recognition. Annu Rev Biochem 53:293–321

Scott JR (1984) Regulation of plasmid replication. Microbiol Rev 48:1–23

Som T, Tomizawa J (1983) Regulatory regions of ColE1 that are involved in determination of plasmid copy number. Proc Natl Acad Sci USA 80:3232–3236

Tomizawa J (1984) Control of ColE1 plasmid replication: The process of binding of RNA I to the primer transcript. Cell 38:861–870

Tomizawa J, Som T (1984) Control of ColE1 plasmid replication: Enhancement of binding of RNA I to the primer transcript by the Rom protein. Cell 38:871–878

Communicated by P. Starlinger

Received November 8, 1985