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DIFFERENTIAL REGULATION OF THE P1 AND P2 PROMOTERS OF THE RIBOSOMAL RNA OPERONS IN *BACILLUS SUBTILIS*

by

David Xiaoming Liu

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

DIFFERENTIAL REGULATION OF THE P1 AND P2 PROMOTERS OF THE RIBOSOMAL RNA OPERONS IN *BACILLUS SUBTILIS*

by

David Xiaoming Liu

Advisor: Professor Rivka Rudner

The expression of ribosomal RNA operons (*rrn*) of *B. subtilis* during fast and slow growth, amino acid starvation and carbon source limitation were examined through the use of single-copy *spoVG-lacZ* fusions integrated at the heterologous *amyE*. The four *rrn* promoters tested, *rrnO*, *rrnJ*, *rrnB*, and *rrnD*, were all growth-rate regulated. Assays of β-galactosidase activity and levels of *lacZ* mRNA showed that solitary P2 promoters were 2-4 times stronger than the solitary P1 promoters for both *rrnO* and *rrnJ* operons. The expression levels also revealed that the promoter upstream region enhances promoter activity. Both P1 and P2 promoters were growth-rate regulated. Only the strong P2 promoters of the *rrnO* and *rrnJ* responded to carbon-source limitation. Their activities were decreased by 70% after 90 minutes of α-methyl glucoside treatment. Both P1 and P2 promoters of *rrnO* and *rrnJ* were subject to stringent condition induced by amino acid starvation. After 60 minutes exposure to 2

mg/ml serine hydroxamate, *rrnO*-P1 and *rrnO*-P2 both decreased by 85% in activity; *rrnJ*-P1 and *rrnJ*-P2 decreased by 60% and 75% in activity, respectively.

The *in vitro* transcription activity of the *rrnO*-P1, *rrnO*-P2, and *rrnO*-P1P2 with or without UAS region was examined using purified *B. subtilis* RNA polymerase. The P2 promoters, in solitary P2 or in tandem P1P2 configuration, were highly active and were insensitive to NaCl concentration change. The P1 promoters, however, whether in separated or in tandem P1P2 form, was equally active to P2 only at very low (10-20 mM) NaCl concentrations. Its activity decreased precipitously as NaCl concentration increased, and was undetectable at 200 mM NaCl. In the natural P1P2 configuration, the P2/P1 ratios were about 1-2 at 10-50 mM NaCl, 10-20 at 100-150 mM NaCl. In solitary forms, the P2/P1 ratios were about 3 at 50 mM NaCl and about 7 at 150 mM NaCl.

The fragments of *rrnO*-UAS, *rrnJ*-P1, *rrnJ*-P2, and *rrnJ*-P1P2 were found in gel retardation assays capable of forming complexes with protein(s) in the protein extract preparations isolated from *B. subtilis* strain IS58 or purified *E. coli* Fis protein. The *rrnO*-UAS binding protein of *B. subtilis* is expressed at the early log phase. Although its expression pattern resembles that of *E. coli* Fis protein, the *B. subtilis* protein shares no homology with *E. coli* Fis protein as judged from immunoblot analysis. It is unclear what role the unidentified *B. subtilis* protein plays in the *B. subtilis* rRNA gene regulation.

Dedication

For love, understanding, encouragement and inspiration

I dedicate this thesis to my father and mother

Acknowledgments

Greatest gratitude goes to my parents, Chenxian and Yufang. Their love, understanding and sacrifice are the inspiration for my graduate study that started 14 years ago in China. Much gratitude also goes to my science parent, Dr. Rivka Rudner, for her guidance and help. Her advice, criticism and friendship are among the most valuable and enduring treasures I ever received.

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I am especially thankful to my aunt Mrs. Yufen Ren for her most generous financial support. The support not only provided me a secured financial footing and allowed me to overcome worries and fears of a debilitating physical condition, it also helped me focus on the future and supplied me plenty of expectation that propelled me forward. I could not overstate the importance of

those tremendous and extraordinary help. In my uphill battle to recover, Dr. Rivka Rudner's husband, Benjamin Rudner, M.D., provided me much professional and personal help. That help was one of the most important components that enabled me to pull through. My greatest respect goes to the members of my advisory committee: Dr. Joseph Krakow, Dr. Marvin Friedman, Dr. David Calhoun, Dr. David Bechhofer and Dr. Rivka Rudner. I must thank them for their kind, candid, and enlightening advice in directing me to complete my graduate study. I am sure that my experience and relationships with them will benefit me even more in the years to come.

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Abbreviations

αMG	α-methyl glucoside
Ac	Sodium acetate
amyE	Amylase gene
Ap^R	ampicillin resistance
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
CAT or Cat	chloramphenicol acetyl-transferase
CIP	calf intestine phosphatase
Cm^R	chloramphenicol resistance
EtBr	ethidium bromide
EDTA	ethylenediamine-tetraacetic acid
Erm^R or Em^R	erythromycin resistance
Fis	factor for inversion stimulation
fis	Fis gene
Glu	glucose
HB-protein	heparin column-bound fraction of crude protein extract
kd	kilodaltons
Kd	dissociation constant for PR_C, the open promoter-RNAP complex
Kf	equilibrium constant for PR_C and PR_O, representing the transition between the two complexes
mM	millimole
MM	minimal medium; (MM1, MM-glu; MM2, MM-Suc; MM3, MM-Ac)
OriC	replication origin
P1	RNA upstream individual promoter
P2	RNA downstream individual promoter
P1P2	RNA tandem promoters
PCR	polymerase chain reaction
pilV	isoleucine-valine promoter of *E. coli*

PMSF	phenylmethylsulfonyl fluoride
PR_C	promoter-RNAP closed complex
PR_O	promoter-RNAP open complex
ppGpp	guanosine 3'-diphosphate 5'-diphosphate
pppGpp	guanosine 3'-diphosphate 5'-triphosphate
RBS	ribosomal binding site
relA$^+$	wild type relA gene
relA$^-$	relaxed relA gene
RNAP	RNA polymerase
r-protein	ribosomal protein(s)
rrn	ribosomal RNA gene or operon
SDS	sodium dodecyl sulfate
SHX	serine hydroxamate
Suc	sodium succinate
TBAB	tryptose blood agar base or TBAB media
TBE	Tris-Boric acid-EDTA buffer
Tc^R	tetracycline resistance
TE	Tris-EDTA buffer
TEMED	tetramethyl-ethylenediamine
trn	tRNA gene or operon
UAS	upstream activating sequence
UP element	defined as the -55 region that interacts directly with α subunit of RNAP
VFCA	vitamin free casamino acid
VY	veal infusion-Yeast extracts
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

INTRODUCTION

The life style of bacteria can be characterized as "feast or famine". As a consequence, bacteria have evolved mechanisms enabling efficient adaptation of their growth-rate to the rapid changes in nutritional supply and other environmental alterations. The synthesis of ribosomal RNAs and ribosomes themselves is finely tuned to the need of cells whose capacity of protein synthesis varies according to the cell growth demands. Bacteria respond to favorable growth conditions by increasing the number of ribosomes in proportion to the cell mass (growth-rate regulation), and they cease the production of new ribosomes when growth conditions are unfavorable (stringent control).

Bacterial ribosomes contain equal number of 23S, 16S and 5S rRNAs as well as over 50 species of ribosomal proteins. Studies have shown that the synthesis of ribosomal proteins (r-proteins) is subjected to a coordinated feedback control regulation. r-proteins are repressors of their own genes and free rRNA molecules relieve the repression by competing with the genes for r-protein binding (Gourse et al., 1985; Yamagishi and Nomura, 1988). Thus, the regulation of rRNA synthesis is the key to understanding the control of ribosome biosynthesis and the mechanisms for cell growth regulation.

1. Organization of rRNA Genes in *B. subtilis*

In general, bacteria have three species of rRNAs (16S, 23S, and 5S rRNAs) whose genes are organized into several transcription units called *rrn*

operons. Each of such operons usually contains one copy of each of the three rRNA genes in the order of 16S-23S-5S. The number of *rrn* operons varies greatly from species to species, though. In the most studied Gram negative bacterium *E. coli*, for example, the number is seven (Kiss et al. 1977). Using integrative mapping analysis, LaFauci et al. (1986) found that the Gram positive *B. subtilis* genome contains 10 rRNA operons. The majority of the 10 operons *B. subtilis* are located near the replication origin, the *oriC* locus; seven of them are clustered between 0° to 20° on the 360° genomic map (Fig.1). Some *rrn* operons are arranged so close to each other that they form doublet (*rrnJ-W*) and triplet (*rrnI-H-G*). All of the *rrn* operons are clustered with groups of tRNA genes. *rrnO* and *rrnA* are the two operons closest to the *oriC* locus and are the only two of the ten *rrn* operons that contain tRNA genes in the 16S-23S intragenic space regions (Stewart and Bott, 1983; Loughney et al., 1982; LaFauci et al., 1986; Ogasawara et al., 1983a). Doublet *rrnJ* and *rrnW* are separated by only 1.2 kb, where 9 tRNA genes are coded (Green and Vold, 1992). In the *rrnI-H-G* triplet, 6 tRNA genes are found between *rrnH* and *rrnG*, while only 300 bp separate *rrnH* and *rrnG*. *rrnB* and *rrnD*, on the other hand, are clustered at their 3' end of the operons with groups of 21 and 16 tRNA genes, respectively. These associations are thought to play an important role in the coordinated regulation of the tRNA and rRNA genes. For example, the 21 tRNA genes clustered with *rrnB* are thought to be transcribed as part of the preceding rRNA genes, although a minor promoter found in the last tRNA gene may be also functional (Green and Vold, 1992; Rudner et al., 1993).

Sequence analysis of the promoter regions have shown that all of the seven *E. coli rrn* operons have a pair of closely arranged promoters (P1-P2) located at the 5' upstream region of the structural genes (Glaser and Cashel, 1979; Jinks-Robertson and Nomura, 1987). Complete DNA sequences of three *B. subtilis* rRNA operons (*rrnO*, *rrnA*, *rrnB*, Ogasawara et al., 1983b; Green et al., 1985) and partial DNA sequences of 5 others (*rrnJ*, *rrnD*, *rrnG*, *rrnW* and *rrnH*) have been determined (Widom 1988; Jarvis et al., 1990; Green et al., 1985; Green and Vold, 1992; Wawrousek and Hansen, 1983). It has been found that the majority of the *rrn* operons in *B. subtilis*, with the exception of *rrnG, rrnH*, and *rrnW*, also contain two tandem promoters (P1 and P2) (Table 1). The two tandem promoters are separated by less than 100 base pairs, with the downstream P2 being located about 100 to 200 base pairs upstream of the 5' end of mature 16S rRNA. Both the P1 and P2 promoters have the canonical -35 sequence and a Pribnow box centered at -10. The regions upstream of their -35 sequence are AT-rich, which is one of the characteristics of very strong promoters (Petho et al., 1986).

Six separate elements in *E. coli rrn* promoter region have been identified that together confer the activity and regulatory properties specific to rRNA synthesis (Gourse et al. 1986; Ross et al., 1994) (Fig. 2a). The six elements are: 1) an upstream activating region (UAS), which contains Fis-binding sites, or Fis-dependent element (Zacharias et al., 1992); 2) Fis-independent element, or UP element, which interacts with the α subunit of RNAP (Zacharias et al., 1992; Ross et al., 1994); 3) the upstream promoter P1, which is the major promoter in

E. coli rrn operons and is subject to growth-rate dependent control and stringent control; 4) a weak downstream promoter P2, which contributes significantly to total rRNA synthesis levels only in slow growth when P1 is repressed; 5) a discriminator, GCGC, located between -10 and +1 of the P1 promoter, which is believed to be a target sequence for stringent control regulation (Travers, 1984); 6) an antiterminator, also called BoxA, a short segment in the *rrn* leader region containing λ *nut*-like sequences, which is required for antitermination (Li, et al., 1984). BoxA allows RNA polymerase to read through the long, untranslated rRNA transcript.

The P1 and P2 promoters in *B. subtilis rrn* operons are similarly arranged. A generalized map of *B. subtilis rrn* operon promoter regions is presented in Fig. 2b. The upstream regions of P1 and P2 are AT-rich and likely contain activating sequences. These sequences may even contain multiple binding sites for a Fis-like regulatory protein as indicated from a sequence comparison between *E. coli rrn* promoters and *B. subtilis rrn* promoters. There are also GC-rich sequences between -10 and +1 in both P1 and P2 promoters, raising the possibility that they function as discriminators in *B. subtilis* just as the GCGC discriminators in the P1 promoters in *E. coli rrn* operons. Besides the apparent similarities, however, the *B. subtilis rrn* operons are clearly more heterogeneous than their *E. coli* counterparts. Ogasawara et al. (1983a) reported that there is a unique inverted repeated sequence immediately after the Pribnow box of *rrnO*-P1 promoter, and one arm of this palindromic sequence appears again in the corresponding position of the downstream P2 of

the same operon, suggesting a competition of alternative base-paring between the two regions. Another unique feature of *rrnO* is that its P1 promoter has 16 bp separating the -35 sequence and -10 Pribnow box, instead of 17 bp in all of the other *rrn* promoters whose sequences are known. It is worth mentioning that *rrnO* happens to be the most active operon in *B. subtilis* (Widom, 1988) and is located very close to *oriC*. There are two other features in the sequences of *B. subtilis rrn* promoters whose significance is not known. One is that there is only one promoter instead of two in the downstream operons in the *rrnI-H-G* and *rrnJ-W* clusters (Fig. 1). The other is that a 65bp insert is located at the 3'-terminus of the leader sequence in *rrnO*, *rrnD*, and *rrnB* operons (Ogasawara et al., 1983b; Stewart and Bott, 1983; Jarvis et al., 1990). Because the three operons do not distinguish themselves as a group from the rest in promoter activities, it is likely that this 65 bp insert, which can form a secondary structure, is not involved directly in modulating transcription regulation, but rather in processing as proposed by Ogasawara et al. (1983).

2. Stringent Response and the Regulation of rRNA gene Expression

When growing bacteria are exposed to a nutritional downshift provoked, for instance, by addition of α-methyl glucoside (αMG) or serine hydroxamate (SHX) to the culture, a transient accumulation of pppGpp and ppGpp (designated as (p)ppGpp, Nishino et al., 1979; Rhaese and Groscurth, 1978) can be observed with a concomitant decrease or cessation of stable RNA (rRNAs and tRNAs) synthesis. This phenomenon is called stringent response

(Cashel and Rudd, 1987) and its mechanisms, though being intensively studied for decades, remains elusive.

Studies have shown that the *relA* gene in *E. coli* (Cashel and Rudd, 1987) and the *rel*A gene in *Bacillus subtilis* (Swanten and Edlin, 1982; Smith et al., 1980) code the enzyme that synthesizes the penta- and tetra-phosphate purines. It was first postulated by Cashel and Rudd (1987), and is now widely accepted that (p)ppGpp is the pleiotropic effector controlling the synthesis of stable RNAs as well as other physiological changes during stringent response. It is believed that (p)ppGpp works at the level of RNA polymerase, causing a preferential decrease of polymerase binding to stringent promoters. Many studies indicated that (p)ppGpp may exert its effect at different levels. It sometimes affects the binding of RNA polymerase to stable RNA promoters (Hamming et al., 1980; Kingston and Chamberlin, 1981); and sometimes it increases the pause time of RNA polymerase at specific sites downstream of *rrnB*-P1 promoter (Kingston and Chamberlin, 1981). It is likely that stringent control is the consequence of (p)ppGpp-induced conformational changes in RNA polymerase which alters its ability to transcribe certain promoters. Although there is a great deal of support for the direct involvement of (p)ppGpp in regulating rRNA synthesis, some controversies persist. Even *in vitro* studies in attempts to demonstrate a direct link between (p)ppGpp and the transcription of rRNA and tRNA have been variable. Some investigators have reported specific inhibition of stable RNA transcription by ppGpp, while others observed little or no effect (Gallant, 1979). In one report, a different nucleotide (ppGp) preferentially inhibited stable RNA synthesis (Nakajima et al., 1982); and in

another, apparently normal stringent responses occurred in the absence of ppGpp accumulation (Pao and Dyess, 1981; Spadaro et al., 1981). These reports indicated that there is still much work to do before assigning (p)ppGpp as the sole effector in stringent control regulation of rRNA synthesis. The difficulties for a clear answer are due to an obvious reason. Growth inhibition predictably accompanies manipulated elevations of (p)ppGpp without nutrient limitation (Sarubbi et al., 1988; Schreiber et al., 1995) and steady-state growth rates achieved in a variety of ways can be correlated with (p)ppGpp levels (Ryals et al., 1982), yet inhibition of growth can occur without participation of ppGpp (Cashel et al., 1996).

It has been found that stringent-controlled promoters have in common a GC-rich region between the -10 Pribnow box and the transcription initiation site. This GC-rich region (5'-GCGC-3'), known as the "discriminator", is highly conserved among stable RNA promoters, and is believed to be the target for the stringent control regulation (Lindahl and Zengel, 1986; Travers, 1984). A single point mutation in this region had been found sufficient to convert the relaxed *E. coli rrnB*-P2 promoter into a stringent-controlled and growth-rate regulated promoter (Zacharias et al., 1989). More systematic mutation studies showed that this GC-rich region is necessary but not sufficient to render a promoter to be stringent control regulated (Gaal et al., 1989; Dickson et al., 1989). The sequences in the core promoters regions (-35 and -10 sequences) are also as important (Josaitis et al., 1995). See Concluding Remarks for more discussion on mutation studies.

3. Growth-Rate Dependent Regulation and rRNA Gene Expression

Ribosomes are composed of three rRNAs and several dozen ribosomal proteins. All of the components of the ribosomes must be made coordinately with each other in different growing conditions. In general, the synthesis of ribosomal proteins (r-proteins) are regulated at the translational level. The mRNAs for r-proteins carry sequences that resemble the binding sites for the rRNA chains, and the r-proteins are synthesized as long as there are vacant binding sites for them on free rRNA chains. However, when present in excess over rRNA to which they bind preferentially, such as in the case of rRNA production cessation induced by stringent control, the r-proteins bind instead to their corresponding mRNAs and thus prevent their own synthesis (Gourse et al., 1985; Yamagishi and Nomura, 1988). Therefore, the rRNA synthesis rate and how it responds to the nutritional environment are the central issues in growth-rate dependent control regulation.

The number of ribosomes in a growing cell fluctuates depending on the environment and cell growth rates (Jinks-Robertson and Nomura, 1987). Different models have been postulated to explain the mechanism of the response of rRNA synthesis to cellular growth rates, which include the direct effector model (Lazzarini, et al., 1971) and the ribosome feedback regulation model (Gourse et al., 1985; Condon et al., 1993). While the two models are not necessarily mutually exclusive, many experiments, especially the gene dosage experiments, have consistently favored the ribosome feedback regulation model. In this model, it was suggested that cells have an inherent capacity to synthesize excess amount of all ribosomal components, but that feedback

regulation of rRNA synthesis by free, non-translating ribosomes prevents the synthesis of more ribosomes. Thus, the amount of ribosomes in cells equals the demand for their capability of protein synthesis. Protein synthesis rates would presumably be dependent on the level of total mRNA available for translation, which is determined by mechanisms such as transcription repression and activation. Although the pathway of the proposed feedback model remains to be elucidated, the region responsible for the feedback inhibition has been located within an *rrn* promoter. By introducing an *rrn* operon on a multi-copy plasmid into a cell lysogenized by λ phage containing an *rrn-lacZ* fusion as a reporter, Gourse et al. (1985) discovered that the sequences around the -35 region of *rrnB* P1 promoter possessed the determinant for feedback inhibition. This is the same region which is required (but not sufficient) for the promoter to be stringently controlled, suggesting the two systems, although not using identical determinants (Josaitis et al., 1995), may nevertheless share some common sequences for their specific recognition. In line with these assessment, Gaal and Gourse (1990) found that ppGpp was not required for growth-rate dependent control of rRNA in *E. coli*. Supporting the different mechanisms involved in growth-rate regulation and stringent regulation, we found in *B. subtilis* that both the *rrnJ*-P1 and *rrnJ*-P2 promoters showed similar growth-rate regulation patterns but different stringent regulation patterns in IS58 (*relA*+) and IS56 (*relA*$^-$) strains (see W. Samarrai's thesis).

Using *rrn*-CAT chromosomal fusions, both at their native chromosomal location and at the λ *att* site, Condon et al. (1992) had shown that all of the 7

intact *E. coli rrn*-P1P2 promoters are growth-rate regulated and are subjected to stringent control. Using an *rrn-lacZ* construct, Widom (1988) were able to examine seven out of ten *B. subtilis rrn*-P1P2 promoters and found all of them are growth-rate regulated in their native chromosomal locations. When expressions of the four *rrn*-P1P2-*spoVG-lacZ* fusions were measured at heterologous *amyE* locus, they showed similar characteristics of being growth-rate regulated. The relative activities of the *B. subtilis rrn* promoters were also in agreement at the two loci. *rrnO* was the strongest, *rrnJ* the intermediate, and *rrnD* and *rrnB* the weakest (Widom, 1988).

4. Differential Regulation of P1 and P2 Promoters in Bacterial Systems

The fact that P1 and P2 are of different strengths and show no obvious sequence homology at the 5' upstream regions had led to the speculation that they may be differentially regulated. In *in vitro* transcription studies, it was found that the upstream P1 promoters of the seven *E. coli rrn* operons are much stronger than their corresponding downstream P2 promoters, although the P2 promoters are relatively more efficient at low concentration of RNA polymerase (RNAP) (Glaser et al., 1983; Sarmientos et al., 1983). Results of *In vivo* experiments have been consistent with the *in vitro* experiments. The P1 promoter is the stronger of the two promoters and is growth-rate regulated; and the P2 promoter is weak and constitutive (de Boer and Nomura, 1979; Lund and Dahlberg, 1979; Sarmientos and Cashel, 1983). Using an *rrn* promoter-terminator fusion plasmid, Sarmientos et al. (1983) showed that *rrnA*-P1 is not only responsive to the growth-rate control regulation it is also the target of the stringent control regulation. In *in vivo*

experiments using *rrn-lacZ* fusion, Gourse et al. (1986) confirmed that P1 is the promoter subject to growth-rate control and have defined the target of the control to a small region around -35 consensus sequence.

Due to the similarity of the overall structure of *rrn* operons in *E. coli* and *B. subtilis*, and that the P1, not the P2 promoters in all the 7 *E. coli rrn* operons are stronger, growth-rate dependent and stringent controlled, it had been speculated that the tandem promoters in *B. subtilis rrn* operons be differentially regulated as well. Ogasawara et al. (1983a; 1983b; 1985a; 1985b) pioneered this research using *rrnO* and *rrnA* promoters. Their S1 mapping experiments appeared to suggest that P2 promoter in *rrnO* and P1 promoter in *rrnA* were preferentially used. However, the rampant RNA processing and degradation made it less convincing that the relative amount of the intact transcripts of P1 and P2 reflected their actual activities. In fact, the correct-size-transcripts of the P1 or P2 promoters only accounted for a minority of the total RNA products. Another attempt to shed some light on the differential regulation of *B. subtilis* P1 and P2 promoters was conducted by Deneer and Spiegelman (1987). They fused the separated *rrnB* P1 and P2 promoters to a promoterless CAT gene and measured CAT gene expression after the constructs were transformed into *E. coli*. Although they demonstrated clearly that, in *rrnB*, P2 is much stronger than P1; and P2, not P1, is growth-rate dependent, the experiments also left some doubt. There are two reasons that one must be cautious in interpreting the results. 1) there is no obvious sequence homology between *B. subtilis rrn* promoters and *E. coli rrn* promoters except the -35 and -10 sequences and; 2) *E. coli* is known to be unreliable for testing promoters of *B. subtilis* genes (Band and Henner, 1984). For instance, the

promoterless *spoVG-lacZ* gene in pDG268, the vector we use in our research, is expressed in its *E. coli* host but remains silent in its *B. subtilis* host. To solve those problems, one must conduct experiments using the *B. subtilis* system.

5. Transcriptional regulatory proteins involved in rRNA gene transcription

The *rrn* promoters are among the strongest in bacteria, contributing more than 50% RNA transcription in fast growing cells (Jinks-Robertson and Nomura, 1987). Their transcription efficiency is known to depend on the superhelicity of the template DNA (Oostra et al., 1981; Yang et al., 1979). In *in vitro* transcription run-off assays, the *rrn* promoters are usually less active than many other gene promoters (Zacharias, 1992), including the *pilV* promoter we used as a control in our experiment. It was not until recently that it was found that Fis protein plays an important role in potentiating *rrn* promoters' high capacity.

Fis is an 11.2 kd, basic protein which binds DNA as a homodimer and was initially identified by its ability to stimulate different kinds of DNA inversion (Haffter and Bickle, 1987; Johnson et al., 1986; Koch and Kahmann, 1986; Bruist et al., 1987; reviewed in Finkel and Johnson, 1992). Subsequently, Fis has been shown to stimulate both excision and integration recombination of bacteriophage λ DNA (Ball and Johnson, 1991a; Ball and Johnson, 1991b; Thompson et al., 1987). It has also been demonstrated that Fis binds the *E. coli* origin of replication (*oriC*) and may play a role in initiation of DNA replication (Filutowicz et al., 1992).

Fis binds DNA in a site-specific manner, although the consensus

sequence for Fis-binding sites (G/TNNYRNNA/TNNYRNNC/A) is highly degenerate (Finkel and Johnson, 1992; Hubner and Arber, 1989). It is likely, however, that in addition to the specificity of nucleotide sequences, Fis may recognize other structural features at or near the binding sites, such as DNA curves (Perez-Martin et al., 1994). Upon binding with its carboxyl-terminal region which contains a helix-turn-helix DNA binding structure (Koch et al., 1991; Osuna et al., 1991), Fis bends DNA by 60^0 to 90^0 at the cognate target sites (Gille et al., 1991; Yuan et al., 1991; Kostrewa et al., 1991; Thompson and Landy, 1988). Mutants of Fis have been identified which are defective in transcription activation, yet their DNA binding and bending characteristics are unaffected (Gosink et al., 1996). It seems that number 71 to 73 amino acids which are in a surface-expose loop between B and C (the B-C loop), and amino acid 74 which is the first amino acid of the helix-turn-helix DNA binding motif may directly contact RNAP (Gosink et al., 1996).

Fis-dependent promoters seem to have some similar features (Verbeek et al., 1990). All of them share a Fis-binding site centered at about -70, and very often they contain additional Fis-binding sites more upstream of the -70 binding site. For example, there are three Fis-binding sites in the UAS regions of both the *rrnB*-P1 and *thrU*(*tufB*) promoters (Ross et al., 1990; Nilsson et al., 1990), and five binding sites at the upstream region of the *fis* gene (Ball et al., 1992). Due to different affinities, the occupancy of these Fis-binding sites may well be dependent on intracellular concentrations of Fis.

The *in vivo* expression pattern of Fis protein fits its role as an activator for rRNA genes. It had been found that Fis autoregulates its own gene

(Ninnemann et al., 1992) and its cellular level in *E. coli* varies dramatically during the course of cell growth and in response to changing environmental conditions (Ball et al., 1992). Upon nutritional shiftup, Fis level undergoes a brief but intense increase (500 times higher at the peak), which is followed by the increase of rRNA production and the exponential phase of cell growth.

The role of the *E. coli* Fis protein in the enhancement of rRNA gene transcription has been demonstrated both *in vitro* and *in vivo*. It had been found that purified Fis activated transcription from *rrnB*-P1 promoters containing Fis-binding site(s) by 10 to 20-fold *in vitro* and had no such effect on promoters containing no Fis binding site (Ross et al., 1990). Promoter fusions in which Fis binding sites were progressively deleted exhibited activity loss *in vivo* accordingly (Ross et al., 1990). Furthermore, mutation at a Fis-binding site not only reduced transcription activity of the *rrnB*-P1 promoter *in vivo*, but also abolished its ability to interact with Fis *in vitro* (Ross et al., 1990).

OBJECTIVES OF THIS STUDY

1. To determine the *in vivo* activities of the P1 and P2 promoters in the *B. subtilis rrn* promoters. We choose *rrnO*-P1P2 and *rrnJ*-P1P2 promoters because *rrnO* was available to us and is the most interesting *rrn* operon due to its special location on chromosome, and *rrnJ* was discovered and its sequence at the promoter region was determined in Dr. Rudner's laboratory.

2. To examine whether the P1 and/or P2 promoters participate, and to what extent if they do, in the growth-rate regulation and in stringent control regulation induced by amino acid starvation and glucose starvation.

3. To investigate how the two adjacent promoters influence one another and whether upstream activating sequence (UAS) exists in *B. subtilis rrn* operons and how significant it is in enhancing P1 and P2 promoters activities.

4. To determine the *in vitro* activities of the P1 and P2 promoters of *rrnO* in solitary as well as in adjacent P1P2 form and study the influence of different NaCl concentrations on the activities of P1 and P2.

5. To investigate the possibility of the existence of an *rrn* transcription activating protein in *B. subtilis*.

MATERIALS AND METHODS

1. Reagents and Enzymes

Bovine serum albumin (BSA), protease, lysozyme, β-mercaptoethanol, DTT, Coomassie brilliant blue, bromophenol blue, X-gal, tetracycline, ampicillin, phenylmethylsulfonyl fluoride (PMSF), and heparin-agarose, were from Sigma; sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide and TEMED from Bio-Rad; restriction nucleases, T4 DNA ligase, DNA polymerase I Klenow fragment and Taq DNA polymerase, from Boehringer Mannheim; DNase I and Vent DNA polymerase, Promega; dATP, dGTP, dCTP, and dTTP from Pharmacia; γ-^{32}P-ATP (3000 μCi/mmole), α-^{32}P-UTP and α-^{35}S-dATP, from Dupont; ECL Western blotting kit from Amersham.

E. coli Fis protein (200 μg/ml in 20 mM Hepes (pH 7.5), 1 M NaCl, 0.1 mM EDTA, 50% glycerol) and rabbit anti-*E. coli* Fis serum (1000X) were kindly given by Dr. Reid Johnson (UCLA; Ball et al., 1992); *B. subtilis* RNA polymerase was kindly provided by Dr. John Helmann (Cornell University; Juang and Helmann, 1994); *E. coli* RNA polymerase was kindly provided by Dr. Joseph Krakow (Hunter College).

2. Plasmid Constructs, Bacterial Strains and Growth Conditions

B. subtilis, *E. coli* strains and plasmids used in this study are described in Table 4. *B. subtilis* strains IS58, IS56 were obtained from Dr. Issar Smith (PHI); SB25 and BD170 were Dr. Rivka Rudner's laboratory collections. The first group of plasmids in Table 4 are cloning vectors (Antoniewkie et al, 1991;

Shimotsue and Henner, 1986). pDH32 (Shimotsue and Henner, 1986) and pDG268 (Antoniewkie et al., 1991) are *B. subtilis* integration plasmids that with slight difference in their sizes and polylinker regions both carry Ap^R and Cm^R genes, a promoterless *spoVG-lacZ* fusion, and two large regions of amylase gene (*amyE*) of *B. subtilis* (Fig. 3a). The second group are the plasmids that contain the original *rrn* promoters and the *veg* promoter. *rrnJ, rrnD* and *rrnB* promoters were cloned in pDG268 by Ann-Marie White from rescued plasmids pGR151 using the enzyme BclI (*rrnJ*, Widom, 1988), pWR305 (*rrnD*, Widom, 1988) and pGS227 from Dr. Keneth Bott (*rrnB*, Deneer and Spiegelman, 1986). *rrnO* promoter was cloned in pDH32 by Dr. Charles Stewart, which originally came from E19 fragment (Ogasawara et al., 1983a). The *veg* promoter was cloned from an unknown gene which is expressed but not growth-rate regulated in the vegetive growth and is not expressed during sporulation (Ollington and Losick, 1985). The third group of plasmids which are mostly separated promoters were designed and constructed for this study.

The molecular sizes and the restriction endpoints for the intact *rrn* promoters are as following: *rrnO*, 1.9 kb, EcoRI-EcoRI; *rrn*J, 1.5 kb, PstI-PstI; *rrn*D, 2.2 kb, HindIII-HindIII; and *rrn*B, 1.8 kb, EcoRI-EcoRI, respectively. The *veg* promoter is 0.48 kb. All of the *rrn* promoters contain portions of the 5'-end of the 16S rRNA genes.

The media used to produce different growth rates and related studies were complex media VY (2.5% veal infusion (Difco) and 0.5% yeast extract (Difco)) and LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 0.01% dextrose), and three supplemented minimal media which were made from a

basal medium (Spizizen minimal salts (see Appendix; Anagnostopoulos and Spizizen, 1961) supplemented with 50 μg/ml of L- tryptophan and 100 μg/ml of L-lysine for IS58 and IS56). MM-Glu: basal medium plus 0.5% glucose, 1% Na-glutamate; MM-Suc: basal medium plus 1% Na-succinate, 0.05% yeast extract, 0.02% vitamin-free casamino acids; MM-Ac: basal medium plus 1% Na-acetate, 0,05% yeast extract, 0.02% vitamin-free casamino acids. In a typical experiment, overnight VY or minimal medium cultures of the various strains containing integrated *rrn-lacZ* fusions were centrifuged, washed and diluted 1 to 25 with fresh, prewarmed media of the three types of media and then shaken at 37°C in 250-ml side-arm flasks. Growth was monitored on a Klett-Summerson spectrophotometer equipped with a red filter. Samples for β-galactosidase assays (2 X 1.0 ml) and for RNA dot blots (2 X 2.0- 4.0 ml) were withdrawn during logarithmic growth at a Klett reading range of 20-250. The stringent response or transcription inhibition was induced to cultures grown in MM-Glu by the addition of 2 mg/ml serine hydroxamate (SHX); 1% α-methyl glucoside (αMG) at a Klett reading of 100. Samples were withdrawn at the time period indicated.

3. Primer Purification and PCR Generation of Promoter Elements

Primers for polymerase chain reaction (PCR) were synthesized using an Applied Biosystems Model 380B DNA Synthesizer. The synthetic oligonucleotides (table 2), supplied at 0.2 μmole amounts, were deprotected after overnight incubation in NH_4OH at 55°C in a sealed vial. The liquids were evaporated in 1.5 ml microtubes in a Savant Speed-Vac centrifuge. Dried

samples were resuspended in 100 µl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) buffer, mixed with tracing dye (50% glycerol containing 0.25% (w/v), BPB and 0.25% (w/v) xylene cyanol), and loaded on G-50 Sephadex column (1x10 ml). Twenty 0.5-0.7 ml fractions were collected while keeping the buffer above the gel bed at all times. The absorbance at 260 nm was determined for all fractions using a Gilford Spectrotometer. The fractions with the peak of absorbance were evaporated in Speed-Vac and the dried samples aliquots were stored at -20°C for later use. The nucleotide sequences of the primers with their target promoter regions and designed end-restriction-sites are listed 5' to 3' in Table 2.

PCR was carried out following procedures of Saiki et al. (1988). In a typical 100 µl reaction, there were 10 ng of forward and backward primers, respectively, 1 to 10 ng template DNA (pPW4 or pAWR118), 5 units of Taq DNA polymerase (Promega), in the reaction buffer (50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 10 mM $MgCl_2$, 200 µM each of dATP, dGTP, dCTP, and dTTP (Pharmacia)) as recommended. Amplification was achieved by 30 cycles of PCR using a 3 minutes (93°C) denaturation for the first cycle, annealing for one minute between 47°C to 58°C (depending on the templates and primers), elongation for 30 to 60 seconds at 72°C, and denaturation for 1 minute at 94°C. The last elongation reaction was at 72°C for 10 minute, followed by cooling to 4°C.

The PCR-created *rrn* promoters used in this study are listed in Table 3 and the sequences for *rrnO* fragments are given in Fig. 20. *rrn*O-P1 is a 240 bp

HindIII-BamHI fragment, extending from -186 to +20 with respect to the +1 transcription start site of *rrn*O-P1 promoter. *rrn*O-P2 is a 341 bp BamHI-BamHI fragment, extending from -84 to +242 with respect to the +1 transcription start site of *rrn*O-P2 promoter. *rrnO*-UAS is a 549 bp EcoRI-HindIII fragment, containing the upstream region of the *rrn*O promoter from -736 to -187 with respect to the +1 transcription start site of *rrn*O-P1 promoter. *rrnO*-P1P2 is a 549 bp HindIII-BamHI fragment, containing the same region as *rrnO*-P1 and *rrnO*-P2 combined. *rrnO*-UAS-P1 is a 754 bp EcoRI-HindIII fragment, containing the same region as *rrnO*-UAS and *rrnO*-P1 combined. *rrnO*-UAS-P1P2 is a 1075 bp EcoRI-BamHI fragment, containing the same region as *rrnO*-UAS and *rrnO*-P1P2 combined. *rrnJ*-P1 is a 194 bp EcoRI-HindIII fragment, extending from -110 to +21 with respect to the +1 transcription start site of *rrn*J-P1 promoter. *rrnJ*-P2 is a 269 bp HindIII-BamHI fragment, extending from -47 to +181 with respect to the +1 transcription start site of *rrn*J-P2 promoter. *rrnJ*-P1P2 is a 433 bp EcoRI-BamHI fragment, containing the same region as *rrnJ*-P1 and *rrnJ*-P2 combined. *rrn*J-P1(H)P2 is a 455 bp EcoRI-BamHI fragment, containing the same region as *rrnJ*-P1P2 but with a 22 bp insertion of direct repeat including a HindIII restriction site between the two promoters due to primer overlap.

4. Cloning of Separated *rrn* Promoter Fragments

PCR fragments of *rrnJ*-P1, *rrnJ*-P2, *rrnJ*-P1P2, *rrnO*-P1, *rrnO*-P2, and *rrnO*-UAS were cut with appropriate restriction enzymes according to the restriction sites designed on the primers from which the fragments were created (Table 2). The promoter fragments were inserted at the polylinker region in pDG268 at the appropriate restriction sites. To optimize the cloning

recombination, the 5'-phosphate groups of the linearized vectors were removed by CIP treatment following the procedure of Ausubel et al. (1989) (see DNA fragment purification, CIP treatment and labeling, Materials and Methods), and a ligation mixture of high vector:target DNA ratio (1:3 to 10) was used. After transformation of *E. coli* JM83 (*pro, thr, supE, F', traD36, proAB, lacI, Z*) with the ligation mixtures, Ap^R *lacZ*+ transformants were selected. The clones were screened first by digestion of plasmid mini-preps with appropriate restriction enzymes followed by verification of their unique sizes shown on agarose gels and polyacrylamide gels. The selected clones were then verified by the expected restriction sites of additional enzymes such as PstI and HindIII which cut at sites on the vector or on the promoter inserts. They were finally confirmed by their ability to turn on the downstream promoterless *spoVG-lacZ* after transformation into *B. subtilis*. The first two PCR-created *rrn* promoters, *rrnJ*-P1 and *rrnJ*-P2, were also confirmed by DNA sequencing after subcloned the separated promoters in pGEM-Cat3Z (Fig. 3). pLR201 was created by joining *rrnJ*-P1 from pLR139 and *rrnJ*-P2 from pLR402 at the HindIII site, and cloning the *rrnJ*-P1(H)*rrnJ*-P2 into pDG268 (Fig. 7).

5. DNA Preparations and CsCl Centrifugation

Plasmid isolation and purification were done following the alkaline procedure (Birnboim, 1983) and CsCl density gradient centrifugation as described by Davis et al. (1986). For large scale isolation, 500 ml LB overnight culture of plasmid-containing *E. coli* strain was chilled on ice and centrifuged. Cell pellet was suspended in 25 ml of 25 ml cold lysis buffer (0.1 M Tris-HCl, pH 7.5, 20 mM EDTA, 20% sucrose). Fifty micrograms of lysozyme was added to

the suspension and gently mixed, followed by the incubation with shaking at 37°C for 15 minutes. Fifty ml of 1% SDS-0.2 N NaOH was added to the centrifuge bottle and it was gently inverted several times. After 10 minutes at room temperature, 37.5 ml of 3.0 M sodium acetate, pH 4.5, was added and mixed with the lysate. The lysate was placed in a -20°C freezer for 1 hour followed by centrifugation at 10,000 rpm in a GSA rotor at 4°C for 1 hour. The supernatant was collected, and mixed with two-volume of 95% ethanol. Plasmid was pelleted by centrifugation and the plasmid pellet was washed twice with 70%. Plasmid was dried and dissolved in 10 ml TE buffer for further purification. Plasmids were purified either by phenol extraction or CsCl-EtBr gradient centrifugation.

6. Preparation of *E. coli* Competent Cells

E. coli strain JM83 was made competent for plasmid uptake by $CaCl_2$ treatment (Hanahan, 1983). Cells were grown at 37°C in a shaker until Klett reading reached 80-100 units. The culture was chilled on ice for 10 minutes and pelleted by centrifugation at 5,000 rpm for 5 minutes. The pellet was resuspended in 0.5 volume of sterile cold 50 mM $CaCl_2$ and repelleted at the same speed and time length. The pellet was gently resuspended in the same volume of cold 50 mM $CaCl_2$ and left on ice for 20 minutes. Competent cells were then centrifuged and resuspended in 0.1 volume of cold sterile 50 mM $CaCl_2$ in 40% glycerol. Aliquots of this competent cell suspension were quick frozen in dry ice-ethanol. Frozen cells were stored at -70°C until use.

For a transformation assay, 0.2 ml of fresh or thawed competent *E. coli* cells were mixed with transforming DNA and were kept on ice for 30 minutes.

The cells were then heat-shocked at 42°C for 90 seconds. One ml LB was added to the cells and the mixture was incubated at 37°C on a roller drum for 90 minutes. Aliquots of the culture were then plated on LB plates containing 100 µg Ampicillin and 0.1ml X-gal (2% X-gal in N', N'-dimethyl formamide).

7. Preparation of *B subtilis* Competent Cells

Competent cells of *B. subtilis* were prepared by the method of Anagnostopoulos and Spizizen (1961). A 0.2 ml aliquot of a daytime grown culture of a single colony was transferred to 5 ml of VY for overnight growth at 37°C in a roller drum. Spot test was done to examine the required genetic markers. The overnight culture was pelleted and resuspended in 1 ml of Spizizen I synthetic media (Spizizen salts supplemented with 0.5% glucose, 0.02% vitamin free casamino acid (VFCA), 0.1% yeast extract, and 0.8% L-arginine), and 100 µg/ml of each required amino acids. In a 250 ml side arm flask 20 ml of Spizizen I medium was brought up to a Klett reading of 20-25 units with the concentrated cell suspension. After 4 to 4.5 hours of growth at 37°C with aeration, the culture was diluted 1:10 in Spizizen II synthetic media (Spizizen salts supplemented with 0.5% glucose, 0.01% VFCA, 0.05% yeast extract, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.05 mM spermine tetrahydrochloride), and 50 µg/ml of each required amino acid, and returned to the 37° C water bath for 90 minutes. Frozen competent *B. subtilis* cells were prepared by centrifugation in sterile centrifuge tubes, resuspended in 1:10 volume of Spizizen II with 5% glycerol, and quick frozen in 2 ml aliquots in a dry ice-ethanol bath. In a transformation assay 0.9 ml of fresh or reconstituted frozen

competent cells was mixed with 0.1 ml of plasmid (5-10 µg) or chromosomal DNA (0.2-2.0 µg), and incubated at 37° C for 30 minutes in a roller drum. DNase I (100 µg/ml in 0.25 mM $MgSO_4$) was added to a final concentration of 10 µg/ml and incubation continued for another 10 minutes. Samples were diluted in dilution saline and plated in 0.1 to 0.2 ml aliquots on appropriate selective plates. Plates were incubated at 37°C for 24 to 48 hours.

8. Selection of Chromosomal Fusions

Purified plasmid DNA or chromosomal DNA with integrated *rrn* promoter-*lacZ* fusions were transformed into variety of *B. subtilis* strains (Table 4) and Cm^R transformants were selected on LB plates containing 100 µg/ml chloramphenicol. Spot tests and replica-plating were conducted to verify the host genetic phenotypes along with the drug resistant markers on the integrated plasmids including *lacZ* trait. The presence of an expected *rrn* promoter or a functioning promoter part can be recognized directly from the blue color or the colonies on X-gal replica-plates. The insertion of the plasmids at *amyE* locus was confirmed by replica-plating on starch plates on which the double crossover (*amyE*⁻) integrants had negative response to Iodine test (flooded with solution of 0.5% I_2-5% KI) while the single crossover (*amyE*+) exhibited an unstained halo of starch hydrolysis. Confirmed integrants were maintained on TBAB (Appendix) agar plates for use.

9. Southern Blotting

Ten µg of *B. subtilis* DNA was digested with EcoRV and loaded on 0.8%

agarose gel for separation. DNA denaturation, gel neutralization, DNA transfer to nitrocellulose membrane, and hybridization, were carried out following procedures of Southern (1975). The probe was a 1.3 kb DNA fragment from the "*amyE* Back" region obtained from an EcoRV-NruI double digestion of pDG268. Southern blot analysis was done by Ann-Marie White and Barbara Studamire.

10. β-Galactosidase Assay

Bacterial strains with integrated *rrn* promoter-*spoVG-lacZ* fusion gene were assayed for β-galactosidase activity by the method of Miller (1974). Two procedures were used in measuring β-galactosidase activity. (1) Duplicates of one-ml cell cultures were taken at varying Klett readings (exact Klett readings are given in Figure legends) . After centrifugation, cell pellets were suspended in 0.9 ml Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) and lysozyme (1mg/ml) was added to the cell suspension. After incubation on ice for 30 minutes, two drops of toluene were added which was followed by immediate vortexing. The tubes were put on ice for 10 minutes to allow the cells to lyse. To make duplicate measurements for each sample, 0.1 ml and 0.2 ml of lysate were taken and mixed with 0.9 ml and 0.8 ml Z-buffer, respectively. These 1.0-ml samples were used for reactions. Reactions were initiated by the addition of 0.2 ml of O-nitrophenyl β-galactoside (ONPG, 4 mg/ml in 100 mM potassium phosphate buffer pH 7.0) and stopped by mixing with 1.0 ml of Na_2CO_3. Cell debris was eliminated by centrifugation. Intensity of yellow color was determined using spectrotometer at 420 nm. (2) Duplicates of one-ml cell cultures were taken at

varying Klett readings (see Figure legends). After centrifugation, cell pellets were suspended in 0.9 ml Z-buffer. Two drops of toluene were added to the cell suspension followed by immediate vortexing. The tubes were put on ice for 10 minutes to allow the cells to lyse. 0.1 ml and 0.2 ml of lysate were taken and mixed with 0.9 ml and 0.8 ml Z-buffer, respectively, which were the samples to use for assays. Reactions were initiated by the addition of 0.2 ml of O-nitrophenyl β-galactoside (ONPG, 4 mg/ml in 100 mM potassium phosphate buffer pH 7.0) and stopped by addition of 1.0 ml of Na_2CO_3. Cell debris was eliminated by centrifugation. Miller units were calculated using the formula: 1 Miller unit =1000 x A_{420} / Vol x Time x Klett x 0.005. The average Miller units for each measurement is the average of two duplicates (0.1 ml and 0.2 ml) and the data used for most of the Figure presentations usually consist two to seven independent experiments.

11. DNA Sequencing

DNA sequencing was done by using the Sequencing kit (USB), double-stranded plasmid DNA as a template, and α-^{35}S-dATP (Dupont, NEN Research Products) as the label. *rrnJ*-P1 and *rrnJ*-P2 promoters were subcloned in pGEM-Cat3Z at the corresponding restriction sites in the polylinker region, and DNAs were prepared as described before. Primers used were either those used for PCR or a universal primer complementary to the polylinker region on the pGEM-Cat3Z vector. DNA sequencing was carried out by Angel Chevrett.

12. RNA Dot Blot and Primer Extension Analysis

The activity of *rrn* promoters at the transcription level was measured by

primer extension analysis using a primer complementary to the *lacZ* gene. RNA dot blot were performed by Walied Samarrai (Samarrai et al., 1996).

13. DNA Fragment Purification, CIP Treatment and Labeling

DNA promoter fragments were released from their respective constructs by digestion using appropriate endonucleases and separated from the vector DNA by electrophoresis on 0.8% agarose gel or low-melting agarose gel. The relevant DNA promoter fragment bands were excised from the gel and further purified using the Gene Clean kit (USB) or direct phenol extraction at 70°C for low-melting gel. Purified DNA fragments were dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and treated with CIP (0.2 U/μg DNA) for 60 minutes in 50 mM Tris-HCl, pH 8.7, 100 mM KCl, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM β-mercaptoethanol (Ausubel et al., 1989). The CIP was removed from the DNA solution by phenol extraction. The DNA was precipitated by ethanol and dissolved in TE buffer before quantified using a UV spectrotometer or a TK100 fluorometer (Hoefer Scientific Instrument).

Phosphorylation reaction mixtures (final volume 30 μl) contained: 100 mM Tris-HCl, pH 9.5, 10 mM $MgCl_2$, 7 mM DTT, 1 mM ATP, 100 μCi γ-^{32}P-ATP, 1.7 μM oligonucleotide and 2 units of T4 polynucleotide kinase. The mixtures were incubated at 37°C for 30 minutes (Ausubel et al., 1989) and then heated at 70°C for 10 minutes to inactivate the kinase. Labeled DNA fragments were separated from free γ-^{32}P-ATP by spinning through a 0.5x5 cm Sephadex-G50 column.

14. Gel-Shift Assay

Gel-shift assays were done following the procedures of Nilsson et al. (1990) and Zacharias et al. (1992) with minor revisions. Reaction mixtures with a total volume of 20 µl containing 10 mM Tris-HCl, pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 100 µg/ml BSA, 6 ng poly dI-dC, 0.3 ng labeled DNA fragments were incubated with varying amounts of either *B. subtilis* protein preparations or purified *E. coli* Fis protein at 37°C or room temperature for 10 minutes. After the incubation, 3 µl tracking dye (50% glycerol containing 0.25% (w/v) BPB and 0.25% (w/v) xylene cyanol) was added and the samples were loaded on an 8% polyacrylamide gel (29:1) in TBE buffer (40 mM Tris-boric acid, pH 8.3 and 2 mM EDTA). The gels were pre-run for 1 hour at 150 V and run after samples were loaded at 200 V for 2-5 hours, dried and subjected to autoradiography.

15. *In vitro* Run-Off Assay

Reactions were performed according to Juang and Helmann (1994) and Nilsson et al. (1990). In 30 µl transcription buffer (20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10% (v/v) glycerol, 8 mM β-mercaptoethanol, 100 µM UTP, 800 µM CTP, 800 µM GTP, 800 µM ATP) containing 0.1-0.3 pmole template DNA, 1-3 µCi ^{32}P-α-UTP, 0.3-1.5 pmole *B. subtilis* RNAP or *E. coli* RNA polymerase (RNAP, based on total protein) and NaCl as indicated. Reactions were initiated by addition of RNAP, incubated (37° C) for indicated time period, and terminated with stop solution (2.5 M NH_4OAc, 20 mM EDTA, and 100 µg/ml carrier plasmid

DNA). After extraction with phenol/$CHCl_3$, the nucleic acids in samples were ethanol-precipitated, and dissolved in 20 µl TE buffer. Transcripts were separated by electrophoresis on denaturing 6% polyacrylamide-7M urea gels and were visualized by autoradiography. Quantitation was done by densitometry using a Molecular Dynamics phosphor imaging system. The molar ratios of transcripts were calculated by correcting for the number of UMP residues incorporated into each run-off transcripts. The OP1 transcript from the P1P2 templates is 345 nucleotides long and contains 108 UMPs; the OP1 transcript from the separated P1 template is 49 nucleotides long and contains 18 UMPs; the OP2 transcripts from the P1P2 and the separated P2 templates are the same, 248 nucleotides long and contains 68 UMPs.

16. Preparation of Crude Protein Extract

Procedures of crude protein extraction were formulated based on the assumption that a regulatory protein similar to *E. coli* Fis (Ball et al., 1992) might exist in *B. subtilis*.

Overnight culture (in MM1) of *B. subtilis* IS58 cells were harvested and inoculated in prewarmed VY to a Klett of 30. The culture was incubated in 37°C shaker. Aliquots were drawn at different growth phases (Fig. 21) and were quickly chilled on ice. Cells were spun down at 4°C for 10 minutes and then quickly frozen in dry-ice bath. After thawed and resuspended in 2 volumes of ice-cold 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 20 mM NaCl, 1 µM phenylmethylsulfonyl fluoride (PMSF), and 10% (v/v) glycerol, the cells were disrupted on a French press for two periods of 10 minutes, with a 5 minutes-

interval (Nachaliel et al., 1989). The suspension was centrifuged at 40,000 g at 4°C for 1 hr, and the supernatant was collected and dialyzed overnight at 4°C against a buffer containing 10 mM Hepes (pH 8.0), 1 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 5% (v/v) glycerol. The resulting crude protein extracts were used directly or further dialyzed against the same buffer containing 50% glycerol. Aliquots of the preps were stored at -70°C.

17. Preparation of Heparin Column-Bound Protein Fraction

Crude protein extracts were placed in a ice slurry and solid $(NH_4)_2SO_4$ was gradually added, with constant stirring, until $(NH_4)_2SO_4$ concentration reached 75%. The precipitated proteins were collected by centrifugation and the protein pellets were dissolved in extraction buffer (20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 20 mM NaCl, 1 µM PMSF, and 10% (v/v) glycerol) and dialyzed first at 4°C for 5 hrs against buffer containing 10 mM Hepes (pH 8.0), 1 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, and 5% (v/v) glycerol, followed with dialyzing against the same buffer containing 50% (v/v) glycerol. Protein preparations were loaded on heparin-Sephadex column (pH 7.6) at a slow pace to let the column absorb heparin-column bound proteins (HB-proteins). The loaded column was washed with sufficient volume of extraction buffer (20 mM Tris-HCl, pH 7.4, 20 mM NaCl, 1 mM EDTA) until unbound proteins were completely eluted. The column was then eluted successively with the binding buffer (10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM EDTA) containing 0.3 M KCl, and the binding buffer containing 1.0 M KCl. Collections were dialyzed against the dialyzing buffer containing 10 mM Hepes (pH 8.0), 1 mM $MgCl_2$, 50 mM NaCl, 1

mM DTT, and 50% (v/v) glycerol. Concentrated protein preparations were stored at -70°C.

18. Determination of Protein Concentration

The protein concentration was determined by the method of Bradford (1976).

19. Western Blotting

Proteins were separated on an 8% SDS polyacrylamide gel and transferred at 2.5 mA/cm^2 for 45 minutes to a 0.1-μm-pore-size nitrocellulose membrane (purchased from Schleicher & Schuell) using a MiniBlot-SDE (Millipore). The western blots (immunoblots) were first incubated at room temperature on rocking bed in 5% milk for 2 hours and then transferred in 5% milk containing 1:1000 diluted rabbit anti-Fis serum (Ball et al., 1992) and incubated for another 2 hours. The membrane was rinsed with TBE buffer and incubated with horseradish peroxidase-labeled goat anti-rabbit. The films were developed using an ECL western blotting kit (Amersham) following the procedure of the manufacturer. Rabbit anti-Fis serum was diluted with 0.1 M NaCl 1000 times and reuse many times as suggested by Dr. Reid Johnson.

RESULTS AND DISCUSSION

1. Construction of *rrn* promoter-*spoVG-lacZ* fusion strains

Although an insertion of an promoter upstream of the *spoVG-lacZ* in pDH32 or pDG268 is needed to turn on the *lacZ* gene expression in *B. subtilis* cells, the promoterless *spoVG-lacZ* is expressed in *E. coli* cells, which makes it impossible to identify target clones directly on X-gal plates. Thus, the identification of cloned *rrn* promoters was made from restriction analyses on mini-preps. The anticipated clones contained DNA inserts of predicted sizes which can be released by the restriction enzymes whose recognition sites were designed at the ends of each of the inserts (Table 2). The functioning of the promoters was confirmed when *B. subtilis* cells bearing these constructs formed blue colonies on X-gal plates.

Using PCR techniques we generated from the cloned *rrnO* and *rrnJ* promoters 10 promoter-element-bearing fragments (Table 3). Seven of them were cloned in pDG268 (Table 4). Two of the promoters (*rrnJ*-P1 and *rrnJ*-P2) and the *rrnO*-UAS fragment were also cloned in pGEM-Cat3Z (Fig. 3b and Table 4). The *rrn-spoVG-lacZ* fusions were introduced into different *B. subtilis* strains by transformation and were integrated at the *amyE* because of the presence of two large segments of amylase gene and the lack of *B. subtilis* functional *oriC* on pDH32 and pDG268. The plasmid integrates as a single copy at the *amyE* locus at 25^0 on the *B. subtilis* genomic map (see Fig. 1, Piggot et al., 1993). The presence of the plasmid in cells was recognized by the Cm^R phenotype which is carried on pDH32 and pDG268.

The integration can be either a replacement recombination or an insertion recombination, though, due to a double cross-over event or, a single cross-over (Campbell-like) event, as illustrated in Fig. 4. The two types of integrants were distinguished by starch-iodine test and Southern blot analysis. In Southern blot analysis, the double cross-over, or *amyE*$^-$, integrants, yielded a single band of the 1.4 kb EcoRV-EcoRV fragment when probed with an EcoRV-BclI "*amyE* back" fragment; while the single cross-over, or, *amyE*+, integrants, yielded the 1.4 kb band and an extra band whose size varied depending on the size of the promoter insert upstream of the *spoVG-lacZ* gene (Fig. 5). In the case of *rrnO* promoter (itself 1.9 kb), for instance, the size of the detected extra band was either 6.1 kb if the cross-over took place at "*amyE* front" region (Fig. 5), or 6.3 kb if at "*amyE* back" region (Fig. 5). To insure predominantly double cross-over, or *amyE*- integrants were selected, the plasmid constructs were linearized by *Pst*I before the transformations were done. *B. subtilis* strains IS58, IS56, BD170 and SB25 were used as recipients for transformation. Transformants were selected on LB plates containing chloramphenicol and then replica-plated on X-gal and starch plates, subsequently. The presence of the individual promoter-*spoVG-lacZ* fusions was detected and confirmed as blue colonies grew on X-gal plates. The *amyE*$^-$ clones were identified on starch plates as they did not exhibit halo of starch hydrolysis after the plates being flooded with iodine solution. In the case of pLR201, which carries the *rrnJ*-P1(H)P2 promoter, its Cm^R *B. subtilis* fusions were mostly white on X-gal replica plates or, occasionally pale blue. The pale blue colonies of pLR203 would produce white ones automatically in subsequent generations on replica-plates

although the drug marker Cm^R associated with the plasmid remained unchanged, indicating the weak and unstable nature of the *rrnJ*-P1(H)P2 promoter. The expression levels of the promoter were determined by β-galactosidase activity assays and measurement of *lacZ* mRNA abundance via RNA dot blot.

2. Expression of P1 and P2 promoters of *rrnO* and *rrnJ* operons

From our early studies using integration plasmid pWR112 (Widom, 1988) we learned that the expression of *rrnO* operon was higher than that of *rrnJ* operon at their respective native loci, and both operons were growth-rate regulated (Fig. 8). In later experiments examining the *rrnO* and *rrnJ* promoters at the *amyE* locus using pDG268 we also showed that the *rrn*O promoter was stronger than *rrnJ* promoter (Fig. 9). At this heterologous locus, the two promoters were also growth-rate regulated: having lowest activity in MM-Ac, higher in MM-Suc, and even higher in MM-Glu. The fact that both *rrnO* and *rrnJ* operons in *B. subtilis* were growth-rate regulated, regardless whether the promoters were located at their native resident loci or at the heterologous *amyE* locus, indicated that the regulatory determinants are carried on *B. subtilis rrn* promoters themselves and are recognized independently of chromosomal locations.

As mentioned in Introduction, the P1 promoters in *E. coli rrn* operons are the major promoters in terms of promoter strength and regulations. In *B. subtilis*, Deneer and Spiegelman's study demonstrated that when measured in *E. coli*,

the P2 promoter of the *B. subtilis rrnB* operon was stronger than the P1 promoter. It is not known, however, whether that would be true if the promoters were tested in *B. subtilis.* To shed light on the question, we examined the expression of the individual P1 and P2 promoters of *rrn*O and *rrn*J integrated in *B. subtilis* strains SB25 and BD170. The activity of the promoters determined by β-galactosidase showed that the P2 promoters in both *rrn*O and *rrn*J are more active than their respective P1. In BD170 the P2/P1 is about 2-4 for *rrnJ* in all the media tested (Fig.10). In SB25 the P2/P1 is about 2 for *rrnJ* both in LB and in MM-Glu (Fig. 11) and about 1.3 for *rrnO* both in VY and in MM-Glu (Fig. 12). The P1 and P2 promoters were also growth-rate regulated. The activities of both *rrnJ*-P1 and *rrnJ*-P2 were about 80% higher in VY than in MM-Glu when the promoters were integrated in BD170 (Fig. 10) and 20-30% higher in SB25 (Fig. 11). The activities of both *rrnO*-P1 and *rrnJ*-P2 were about 20-25% higher in VY than in MM-Glu when the promoters were integrated in SB25 (Fig. 12). In Fig. 10, we also see an 80% higher promoter activity for *rrnJ*-P1P2(-UAS) in VY than in MM-Glu. For the *rrnO*-P1P2 with UAS, its β-galactosidase activity in SB25 was about 10% higher in VY than in MM-Glu (Fig. 12). In *B. subtilis* strain BD170, however, the activity of the *rrnJ*-P1P2(+UAS) was uncharacteristically lower in VY than in MM-Glu (Fig. 10), which is likely due to fast cell-autolysis and poor cell harvest in VY. Actually, on many occasions and in several different *B. subtilis* strains, the degradation in VY was so visible that the cells were often lysed before harvesting and therefore RNA measurement was not done.

Other observations are that the combined activities of *rrnJ*-P1 and *rrnJ*-P2 is about equal to the activity of *rrnJ*-P1P2 in BD170; and that the *rrnJ*-P1P2 with UAS is about 2-4 times more active than *rrnJ*-P1P2 without UAS (Fig. 10). In SB25, the activity of *rrnO*-P1P2 with UAS is less than the combined activities of *rrnO*-P1 and *rrnO*-P2 (Fig. 12). These data not only suggested that the UAS region contains *cis* activating element(s), but also indicated that when separated the combined activity of P1 and P2 is likely more than their combined activity when they are in tandem P1P2 configuration, presumably due to elimination of promoter occlusion.

The relative activity of the P1 and P2 promoters in MM-Glu was also measured by primer extension using the intact *rrn* promoters (Samarrai et al., 1996). With a downstream primer which complements the start of the *lacZ* gene, we measured the relative *in vivo* abundance of P1 and P2 transcripts and found the P2/P1 ratio for *rrnO* was 10, and for *rrnJ*, 12 (Samarrai et al., 1996). These P2/P1 ratios are much higher than the P2/P1 ratios for separated P1 and P2 promoters, which is another indication that separated P1 promoters may be more active than they are in P1P2 tandems. In *E. coli*, the P1 promoter is the dominant promoter in the *rrn*-P1P2, and the P1 promoter has an occlusion effect on the P2 promoter which can be eliminated when P2 is separated (Gafny et al., 1994). We suspect that a similar occlusion mechanism existed in the *B. subtilis rrn* P1 and P2 promoters. An alternative explanation is that the closely aligned P1 and P2 promoters compete for the limited amount of RNAP in cells, resulting to the further weakening of the inherent weaker P1 promoters, increasing the apparent differences of the two promoters.

The core promoter of the *E. coli rrnB*-P1 is activated 20- to 30-fold by its UAR region, a bent DNA consisting Fis-dependent and Fis-independent *cis* elements (Plaskon and Wartell, 1987; Ross et al., 1990; Leirmo and Gourse, 1991), stretching from -51 to -150 with respect to the transcription start site of the P1 promoter (Gourse et al., 1986). The -150 to -500 region of *rrnB* contains an additional 5 to 10% activation activity (Gourse et al., 1986). Comparing the expression levels of *rrnJ*-UAS-P1P2 and *rrnJ*-P1P2, we saw 2-4 folds stimulation in activity due to the extra 1.1 kb UAR region (Fig. 10). Because *rrnJ*-P1P2 contains not just the core promoters of P1 and P2, but also considerable upstream sequences which contains potential UP element, a smaller stimulation by the extra 1.1 kb UAR was expected. Furthermore, like the -150 to -500 region in the *E. coli rrnB*-P1 (Gourse et al., 1986; Josaitis et al., 1995), this 1.1 kb UAR did not change promoter's ability in responding to growth-rate regulation, suggesting the sequence requirements for growth-rate dependent regulation in *B. subtilis rrn* operons may also located near the core promoter regions.

In an attempt to understand the relationship between the adjacent P1 and P2 promoters, we introduced a 22-bp insertion between P1 and P2 (Fig. 13) by joining the separated *rrnJ*-P1 and *rrnJ*-P2, forming pLR201. This *rrnJ*-P1(HindIII)P2 promoter has only 14% of the activity of the *rrnJ*-P1P2 promoter (compare pLR203 to pLR201 in Fig. 7), although the possible UP element centered at the -55 region was obviously untouched. Thus, we showed that a insert of 22 bp in the intervening sequences between the two adjacent promoters severely impedes the functioning of both the upstream and the

downstream promoters. This unexpected consequence suggested that the intervening sequence between P1 and P2 do not simply act as a spacer separating P1 and P2, it also provides some kind of functioning role in addition to the normal activating sequences normally found at the 5' upstream region of promoters. One possibility for the loss of activity of both promoters is that the spacer region is acutely bent and the 22-bp insertion makes the bent region even more significant that the two promoters in the proximity are no longer accessible to RNAP and transcriptional apparatus. A classical example which can illustrate the very delicate nature of this kind is the formation of DNA loop in the *E. coli* arabinose operon (*araC*). A five-base-pair insertion at the upstream region of *araC* can introduce half a helical turn and prevent the contact of two DNA-binding proteins which normally interact with each other to form a DNA loop (Schleif, 1986). The 22-bp insertion in *rrnJ*-P1(H)P2 is likely to introduce about two helical turns in the intervening sequence region between P1 and P2, but the position of the curved DNA may be so changed that both the P1 and P2 promoters are no longer accessible to RNAP molecules.

3. Response of *rrn* Promoters to stringent condition

3.1. Carbon source starvation Carbon-energy source starvation can be induced by α-methyl glucoside (αMG), a glucose analog which competes with glucose but is unable to be metabolized by bacteria (Hagihira et al., 1963). Addition of αMG to growing bacterial cultures also elicits the accumulation of (p)ppGpp in wild type *B. subtilis* (Nishino et al., 1979; Gropp et

al., 1994).

To see how individual P1 or P2 promoters in the *rrn* promoters respond to glucose exhaustion, the bacterial strains containing integrated solitary promoter-*spoVG-lacZ* fusions were treated with αMG (1%). As shown in Figs. 14 and 15, after 90 minutes exposure to 1% αMG, the amount of *lacZ* mRNAs in both the *rrnO*-P2 and the *rrnJ*-P2 cells decreased 70-80% in the *relA*$^+$ background. The *rrnO*-P1 and *rrnJ*-P1 promoters, on the other hand, both remained relatively unaffected. In the *relA*$^-$ background, all of the promoters of *rrnO* and *rrnJ* continued to function, reaching levels of 122-140% of the untreated samples.

The effect of αMG on the expression of the four *rrn* promoters and the *veg* promoter was similarly examined and the results are shown in Fig. 16. After 90 minutes of addition of αMG (1%) to the cultures the levels of *lacZ* mRNA transcribed from *rrnO*, *rrnJ*, and *rrnB* in the *relA*$^+$ background dropped to 10-20% of the untreated controls. The *veg* promoter, and most likely the *rrnD* as well, did not respond to the αMG, showing similar levels of *lacZ* mRNA before and after the treatment. We concluded from the above experiments that at least three of the four *B. subtilis rrn* promoters tested (*rrnO*, *rrnJ*, and *rrnB*) are subject to stringent control regulation elicited by glucose starvation. Because the low activity of *rrnD* and the lack of decreasing pattern as manifested in *rrnO*, *rrnJ*, and *rrnB*, we believe that the response of *rrnD* to glucose starvation can not be decided until additional experiments are done. We also learned that the P2

promoters in both *rrnO* and *rrnJ* respond to αMG-induced stringent condition but the P1 promoters are insensitive to the treatment.

3.2. amino acid starvation It is well known that ribosomal RNA synthesis is essentially switched off upon the accumulation of uncharged tRNAs induced by the addition of an aminoacyl hydroxamate or O-methyl threonine to growing cultures (Cashel and Rudd, 1987; Nishino et al., 1979). In both *E. coli* and *B. subtilis*, the addition of the amino acid analog to bacterial cultures promotes transient accumulation of (p)ppGpp and inhibits the synthesis of stable RNA in a *relA*$^+$ strain but not in the relaxed strain (Sarmientos and Cashel, 1983; Smith et al., 1980; Nishino et al., 1979).

We looked at the effect of serine hydroxamate (SHX; 2mg/ml)-induced amino acid starvation on the expression of solitary P1 and P2 of *rrnO* and *rrnJ* operons. RNA dot blot quantitation of the *lacZ* mRNA before and after amino acid starvation is shown in Fig.17. The expression of both the *rrnO*-P1 and *rrnO*-P2 promoters decreased approximately 85% in strain IS58, the wild type strain. As expected, in strain IS56 (*relA*$^-$) transcriptions were undisturbed for both P1 and P2 promoters. Similar but less sensitive responses were recorded for the *rrnJ*-P1 and *rrnJ*-P2 promoters (Fig. 18), which showed 45% and 75% decreases for P1 and P2, respectively. In the relaxed strain (IS56), they continued to function in the presence of the inhibitor.

We also looked at the effect of SHX-induced amino acid starvation on the synthesis of *lacZ* mRNA from the four intact *rrn* promoters and the *veg* control promoter. As shown in Fig 19, in the *relA*$^+$ strain (IS58), the activity of *rrnO* and

rrnJ, the two strong promoters, decreased sharply by 90% within 60 minutes, while the weak promoters *rrnD* and *rrnB* seemed insensitive to the treatment. The unregulated *veg* promoter was insensitive to the presence of SHX and continued to function as expected. When the P1 and P2 promoters in their natural P1-P2 configuration were examined for responses to SHX treatment, primer extension analysis showed that both P1 and P2 in *rrnO* decreased their activities after addition of SHX to the cultures (see Samarrai et al., 1996), indicating that the P1 and P2 promoters behaved consistently in response to amino acid starvation no matter whether they are solitary or in a P1P2 tandem configuration.

Two more important observations should be pointed out. First, amino acid starvation in general seemed to produce sharper responses from the *B. subtilis rrn* promoters than glucose starvation. This is significant because we found in a recent study that the level of cellular pppGpp after 10 minutes of SHX treatment was 0.75 nmole/A_{690}, while that after 10 minutes of αMG treatment was 0.43 nmole/A_{690} (Samarrai et al., 1996). In other word, the pppGpp level during amino acid starvation was about 70% higher than during glucose starvation. These results are consistent with the assertion of a simple inverse relationship between the levels of (p)ppGpp and the levels of *rrn* promoter activities. The *rrnB* seemed to be an exception. But as discussed earlier, it is conceivable that the *rrnB* data could be an aberration caused by its low activity. Secondly, it seemed that the *B. subtilis rrn* promoters are more diverse than *E. coli rrn* promoters in response to stringent control and the stronger *B. subtilis rrn*

operons (*rrnO* and *rrnJ*, for example) seemed extremely sensitive to stringent condition. The decrease in activity for the seven *E. coli rrn* promoters during amino acid starvation was about 2-fold (Condon et al., 1993). The *B. subtilis rrnO* and *rrnJ* promoters, for example, had about 10-fold decrease during amino acid starvation, whereas the *rrnD* and *rrnB* promoters were indifferent to the treatment.

4. Preliminary studies on a *B. subtilis* Fis-like protein

In *E. coli*, Fis protein is a stimulatory transcription factor for rRNA gene expression. We have observed in *B. subtilis* the coupling of the increase of cell growth-rate and the increase of *in vivo* expression of *rrn* promoters. We wanted to see whether a Fis-like protein is involved in the process in *B. subtilis*. We first examined the sequences of various *B. subtilis rrn* promoters for the Fis-binding consensus sequence (G/TNNYRNNA/TNNYRNNC/A, see Hubner and Arber, 1989; Finkel and Johnson, 1992). In the 5'-upstream region of *rrnO* we found 13 regions which matched the consensus sequence of a Fis binding site (Fig. 20). Therefore, should there be a Fis-like protein in *B. subtilis* it could be detected in a gel-shift assay using a DNA fragment containing Fis binding sites. Crude protein extracts were prepared from cells harvested at different time during growth as indicated in Fig. 21, and were mixed with ^{32}P-labeled *rrn*O-UAS for 15 minutes at room temperature before loading on 8% polyacrylamide gels for separation. The autoradiogram (Fig. 21) revealed that there were binding properties in the protein preparation isolated from early log phase, which is somewhat similar to that of Fis protein in *E. coli* where Fis is expressed

in the fastest growing cells (Ball et al., 1992). Another binding property was detected in the #8 fraction, which may be caused by excess nucleases (more likely RNases) produced at late log phase. We found, for example, that heparin-Sephadex column enriched #8 protein preparation (HB-preparation) contained enough RNases to degrade all the transcripts when it is added to an *in vitro* transcription assay system (data not shown).

To find the best binding condition, we tested the binding activities using different concentrations of the #3 HB-protein preparation, which was isolated from early log phase and possessed the highest affinity to *rrnO*-UAS fragment (Figs. 21). In Fig. 22, we showed (lanes 8-10) that the *rrnO*-UAS fragment (with equal molar vector plasmid pGEM-Cat which also had a band at the top of the gel) and the #3 HB-protein formed two complexes (C1 and C2) which migrated slower than the free DNA band (F). These two complexes appeared on the gels simultaneously when 0.2 μg of HB-protein preparation was mixed with *rrnO*-UAS fragment (lane 9), and the number and positions of the bands remained the same when 0.5 μg of the HB-protein was used (lane 10). Thus, different concentrations of binding protein did not change the band pattern. These data indicated that there were likely only two binding sites on the 549 bp *rrnO*-UAS fragment for the *B. subtilis* protein in the #3 protein fraction. We also tested other protein preparations in the gel shift assay. After the Heparin-column step, the #6 preparation, for example, produced DNA smears (Fig. 22, lanes 5, 6, and 7). Because no free *rrnO*-UAS DNA band could be seen, the DNA smears seemed more like the result of degraded vector plasmid than shifted *rrnO*-UAS

fragment. The *E. coli* Fis protein, which was used as a control, generated multiple bands (Fig. 22, lanes 3 and 4). The number of those multiple bands increased and their migrations slowed as Fis concentration increased, indicating that more Fis molecules would bind to the *rrnO*-UAS DNA at higher Fis concentration. We interpreted the above results as follows: 1) the 549 bp *rrnO*-UAS fragment contains sequences which can interact with both *E. coli* Fis protein and a *B. subtilis* protein, suggesting the existence of a Fis-like *rrnO*-UAS-binding protein in *B. subtilis*; 2) the *B. subtilis* protein seems more selective than *E. coli* Fis protein in binding to *B. subtilis rrnO*-UAS DNA; it formed two complexes with the DNA fragment in contrast to several bands (more than 6) formed between *E. coli* Fis protein and *rrnO*-UAS, which was dependent on the concentration of Fis protein.

We also tested the ability of HB-protein preparation of *B. subtilis* proteins to bind to other promoter fragments. In *E. coli*, there are 3 binding sites in the UAS of *rrnB*-P1 promoter, and the closest binding site to the P1 promoter is centered at -70 with respect to the +1 (Ross et al., 1990; Nilsson et al., 1990). The separated *rrnO*-P1 or *rrnJ*-P1 promoters contain, besides the core promoter and the UP element, about 150 bp 5' upstream sequence with respect to the P1 promoter. The *rrnO*-P2 and *rrnJ*-P2 promoters also contain portions (about 40 bp) of its UAS region. As shown in Fig. 20, *rrnO*-P2 fragment contains a putative Fis site centered at -65, comparable to the location of -70 in most *E. coli* Fis-site-containing promoters. The *rrnO*-P1 fragment contains two Fis-like sites, one at -45, the other -175, with respect to the transcription start site of P1. Since DNA foot printing was not done, we do not know yet whether these sequences

are the target to which the *B. subtilis* protein binds.

The gel-shift assays using *rrnJ*-P1, *rrnJ*-P2 and *rrnJ*-P1P2 showed that these promoter fragments were capable of interacting with *E. coli* Fis protein (Figs. 23 and 24). Because *E. coli* Fis protein is known to be able to bind DNA in a non-specific fashion and binds to its target sites with higher affinities, we tested the relative binding affinity of Fis to a 315 bp BamHI-HindIII fragment from *B. subtilis* 23S *rrn* structural gene in pYR104 (Jarvis et al., 1990), which is unlikely to contain any regulatory DNA sequence. We showed that it took over 10 times more Fis protein to generate similar DNA shifting pattern for the 23S rRNA fragment than for the *rrnO*-UAS fragment (Fig. 25). In fact, Fis at concentrations that shift the *rrnO*-UAS (0.3 ng), *rrnJ*-P1 (0.2 ng), *rrnJ*-P1P2 (0.2 ng), *rrnJ*-P2 (0.6 ng), and 23S gene (0.4 ng) are: <2 ng, <2 ng, about 2 ng, 0.8-2 ng, and about 20 ng (Figs. 24 and 25), respectively. Thus, the interaction between *E. coli* Fis protein and *B. subtilis rrn* promoter fragments seemed to be a more specific binding, at least by a factor of 10.

Western blot analysis was used to see if the *B. subtilis* DNA binding protein(s) share similarities with *E. coli* Fis. Using anti-*E. coli* Fis serum, we identified two distinct protein bands on the Western blot membrane whose molecular weights were estimated at about 45 and 65 kd, respectively (Fig. 26a, b). As a control, the purified *E. coli* Fis protein formed a single band at about 12 kd, which corresponded to the single protein band on protein gels visualized after stained with Coomassie blue (compare Fig. 26b, c). Since the two protein bands are present in many samples of protein extracts, they do not seem to correspond to the *rrnO*-UAS-binding protein in the #3 extract. Our preliminary

conclusion is that there is at least one *B. subtilis* protein that can bind *rrnO*-UAS and is present in a much higher concentration at early exponential growth stage. It is unlikely, however, that the *B. subtilis* protein is homologous to *E. coli* Fis protein. Neither one of the two proteins (45 and 65 kd) recognized by anti-*E. coli* Fis serum is likely to be the protein forming the DNA-protein complexes in the gel-shift experiments.

5. *in vitro* Transcription of *B. subtilis rrn* promoters

Using PCR-created *rrn* promoter fragments (Table 3), we examined the *in vitro* transcription activities of the P1 and P2 promoters of *rrnO* and *rrnJ*, employing *B. subtilis* and *E. coli* RNA polymerases. We examined the effect of NaCl concentration on the transcription of P1 and P2 promoters on the *rrnO*-P1P2 template. As shown in Fig. 27, both P1 and P2 were active at the low end of NaCl concentration. Actually, there was little difference in activity between the two at 10 mM and 20 mM NaCl and the P2/P1 activity ratio was about 1. As NaCl concentration progressively increased, however, the difference between the two promoters became dramatic (Fig. 27). While transcription from P1 dropped more than 95% at 150 mM of NaCl and became undetectable at 200 mM of NaCl, the P2 promoter maintained a consistent level of transcription at both high and low NaCl concentrations (Fig. 27c). Therefore, P1 is a NaCl-dependent promoter whose activity drops precipitously as NaCl concentration rises and P2 is a NaCl-independent promoter which remains active at high and low NaCl concentrations.

At low NaCl concentrations, the UAS region carried on the *rrnO*-UAS fragment seemed to have little effect on the relative strength of the P1 and P2 promoters in the tandem P1P2 context. At 50 mM NaCl, for example, the P2/P1 ratios were about 1.9 to 2.1 for the P1P2 with or without the UAS region (Fig. 28). The ratio was slightly higher, or 3.5, for the separated P1 and P2 promoters (Fig. 30, 50 mM). At 150 mM of NaCl, however, the P2/P1 ratios were generally higher, ranging from 11.5 and 7.3 for *rrnO*-P1P2(-UAS) and *rrnO*-P1P2(+UAS) (Fig. 29), to 6.7 for the separated P1 and P2 promoters (Fig. 30, 150 mM). Thus, higher NaCl concentrations generally increases the difference between the *rrnO*-P1 and the *rrnO*-P2 promoters.

The activity of the P2 promoter was quite high and consistent under a wide range of NaCl concentrations, in solitary or tandem P1P2 forms, and with or without UAS (Figs. 28, 29 and 30). This consistency suggests that the P2 promoter was expressed at about its maximum activity under the experimental conditions.

The activity of the P1 or P2 promoters on the *rrnJ*-P1P2 fragment was compared with the promoters on the *rrnJ*-P1(H)P2 fragment. At 10 mM NaCl, the P1 and P2 on *rrnJ*-P1P2 were quite similar in activity while both promoters on *rrnJ*-P1(H)P2 were undetectable (Fig. 31). This is consistent with the *in vivo* experiments where the *rrnJ*-P1(H)P2 promoter was about 14% of the activity of *rrnJ*-P1P2 (Figs. 7 and 13). Since the separated *rrnJ*-P1 and *rrnJ*-P2, from whom the *rrnJ*-P1(H)P2 was formed, seemed to function normally both *in vivo* and *in vitro*, the loss of activity of the two

promoters in *rrnJ*-P1(H)P2 is most likely due to stearic hindrance created by the 22-bp insertion. The DNA sequence between the P1 and P2 promoters in *rrnJ* contains stretches of purines, including polyadenines (Widom, 1988), which promote curved DNAs (Galas et al., 1985; McAllister and Achberger, 1988). Intrinsic bend DNA by itself can stimulate transcription several fold *in vivo*, mimicking the CAP activation (Bracco et al., 1989); it can also decrease the strength of promoters by impeding promoter clearance (Ellinger et al., 1994). We suspected that a unique geometry (distribution of shape and strength of hydrogen bonds of the DNA double helix) between the closely arranged P1 and P2 promoters is required for RNA polymerase binding and the promoter clearance, and the 22-bp insertion interrupted or distorted the non-linear structure, repressing the activity of both P1 and P2 promoters.

The RNAP in *B. subtilis*, although containing an extra δ subunit, is structurally and functionally similar to its counterpart in *E. coli*. We compared the transcription of the P1 and P2 promoters on *rrnO*-P1P2 fragment using purified RNAPs. As shown in Fig. 32a, the pattern of the transcriptions at 150 mM NaCl by *E. coli* RNAP and *B. subtilis* RNAP were quite similar. Both produced much more P2 transcripts than P1 transcripts. While the data indicated that the *E. coli* RNAP can still recognize the two *B. subtilis* promoters, they also revealed subtle differences. The *E. coli* RNAP produced about 50% more transcripts from the weak P1 promoter and about 25% less from the stronger P2 promoter than the *B. subtilis* RNAP did

(Fig. 32b, c). Consequently, the ratio of P2/P1 transcription became 9 for the *E. coli* RNAP, compared to about 20 for the *B. subtilis* RNAP.

CONCLUDING REMARKS

While there have been extensive studies on the growth-rate dependent regulation and stringent regulation of the rRNA genes in *E. coli*, there have been very limited reports in *B. subtilis*, the most studied Gram positive bacterium. In this study, we examined the expression of the P1, P2 and P1P2 promoters of the *rrnO* and *rrnJ* in *B. subtilis* at the *amyE* locus. The *rrn* expression on complex and on different minimal media shows that the ribosomal promoters differ significantly from one another in strength. The *rrnO* promoter is the strongest, *rrnJ* is less strong than *rrnO*, and *rrnD* and *rrnB* are the weakest. The order of promoter strength correlates to a certain extent with their genomic locations relative to the *oriC* (Widom, 1988). Both *rrnO* and *rrnJ* promoters are growth-rate regulated at the heterogenous *amyE* locus, resembling the pattern of the two promoters at their native locations (Widom, 1988). Thus, the growth-rate regulation determinants carried on the *rrnO* and *rrnJ* promoters function independently to chromosomal locations.

We compared the effect of SHX and αMG on the expression of four *rrn* promoters (*rrnO, rrnJ, rrnD, rrnB*) and the *veg* promoter. The two strong *rrn* promoters (*rrnO* and *rrnJ*) were stringently regulated, exhibiting an 8-15 fold decrease in expression upon amino acid and carbon-source starvation. The weak *rrn* promoters (*rrnD* and *rrnB*) seemed much less sensitive to both treatment. In fact, only *rrnB* seemed to have shown a clear response to glucose starvation. Since the activities of *rrnB* and *rrnD* were quite low, their responses

to SHX and αMG in Figs. 16 and 19 should be considered preliminary. In *E. coli*, all seven operons are stringently regulated, exhibiting almost a uniform 2-3 fold decrease upon SHX treatments (Condon et al, 1992). It is evident from our study that the *B. subtilis rrn* operons are more diverse in terms of their activity, response to growth-rate regulation and to stringent conditions.

The DNA targets for stringent regulation have been studied extensively with both rRNA and tRNA genes in *E. coli.* A highly conserved GC-rich region known as discriminator, between the -10 region and the transcription start site, has been noted as a common feature of promoters regulated by stringent response (Travers, 1984; Zacharias et al., 1989). In the seven *E. coli rrn* operons, the consensus discriminator sequence GCGC is present in all of the P1 promoters which are stringent controlled, and absent in all of the P2 promoters which are not stringent controlled (Zacharias et al., 1989). A single base substitution (A to G at position -6) that introduced GCGC sequence at the location as it appears in P1 promoters converted the *E. coli rrnB* -P2 promoter to that of stringent controlled as well as growth-rate regulated (Zacharias et al., 1989; 1991). Mutagenesis studies on the core promoter region (-35 and -10 regions) revealed that stringent control and growth-rate dependent control do not have exactly the same sequence requirements (Gaal et al., 1989; Dickson et al., 1989; Josaitis et al., 1995). Gourse and coworkers identified several *rrnB*-P1 mutants, including a double base-pair substitution [C-1T, C-15G], a single base-pair substitution [C-1T], an insertion creating a consensus 17-bp spacing between the -10 and -35 hexamers [T ins -23], which are all not growth-rate

dependent regulated but stringent regulated. No mutants which were growth-rate regulated but not stringent regulated were found. The authors believed that this might be the consequence of the way the mutants were selected (Josaitis et al., 1995). From these studies and studies on the UP elements and UAS region, it seemed that 1) the -35 and -10 regions contain the determinants for both the stringent control and growth-rate regulation; 2) the discriminator also contains necessary (but insufficient) determinants for stringent regulation; 3) the UP element interacts with the α subunit of RNAP, increasing the basal level activity of the core promoter; and 4) the UAS region provides anchors for Fis protein binding, functioning as enhancers.

In the promoter regions of at least the seven sequenced *rrn* operons of *B. subtilis* (Ogasawara et al., 1983a; Green et al., 1985; Jarvis et al., 1990; Widom, 1988), a consensus sequence motif (GTC/TGC/TT) can be found around +1 region in both P1 and P2 promoters. This sequence motif has been considered as the discriminator in *B. subtilis rrn* operons (Ogasawara et al., 1983a) and is believed to be the target for stringent control regulation (although it has not been proven). Our results showed that both P1 and P2 promoters are capable of responding to stringent condition induced by SHX, supporting the assumption that the conserved discriminator sequences function as the determinant for stringent control regulation in *B. subtilis rrn* promoters.

We have compared the effects of SHX and αMG on the expression of four promoter elements of *rrnO* and *rrnJ* fused to a *lacZ* reporter gene. Unlike the situation in *E. coli* where only P1 is stringent response controlled, both

promoters (P1 and P2) were capable of responding to SHX. On the other hand, the two promoters responded differently to carbon starvation. The activity of the downstream promoter (P2) was turned off while the P1 activity persisted. Because amino acid starvation induces pppGpp and ppGpp levels 70% and 40% higher than glucose starvation does (Samarrai, et al., 1996), one theory to explain the differences between the P1 and P2 promoters in response to amino acid starvation and glucose starvation is that both promoters can respond to higher levels of (p)ppGpp while only P2 is sensitive enough to lower levels of (p)ppGpp. In *E. coli*, only upstream P1 promoters in the *rrn* operons respond to amino acid or glucose starvations while the downstream P2 promoters are not stringently controlled (Gafny et al, 1994; Glaser et al, 1983). In the absence of P1, however, the solitary *E. coli rrnA*-P2 promoter was found to be a much stronger promoter than it is in the P1P2 context and it was also found to be stringently controlled (Gafny et al, 1994). They also found that even the strong P1 promoter will be weakly expressed if it is placed downstream of another P1 in an artificial P1P1 configuration. Gafny et al. (1994) proposed that the downstream promoter being weakly expressed is the result of an occlusion effect by the adjacent upstream promoter. We noticed that in our data, the P2/P1 ratios of transcriptional activities were 3 to 4-fold higher when the P1 and P2 were in their natural tandem context than in solitary forms. Here, we also see an apparent inhibition to the weaker P1 promoter (upstream) by the stronger P2 promoter (downstream) when the two promoters are arranged in P1P2 configuration. It could be the result of a similar occlusion effect.

The strength of the P1 and P2 promoters of *B. subtilis rrn* operons and their differential response to growth-rate are also different from those of *E coli.* In *E. coli*, P1 promoters are much stronger than P2 promoters and only the P1 promoters are growth-rate regulated. In *B. subtilis*, we found that the P2 promoters in *rrnO* and *rrnJ* operons are more active than their respective P1 promoters but both P1 and P2 are growth-rate regulated. It had been reported in an early study (Deneer and Spiegelman, 1987) that only the P2 promoter of the *B. subtilis rrnB* operon was growth-rate regulated. Deneer and Spiegelman (1987) separated the P1 and P2 promoters of *rrnB* and cloned them in pKK232-8, with a Cat reporter gene, and examined the promoters in *E. coli.* Although it is possible that the P1 promoter in *rrnB* behaves differently from the P1 promoters in *rrnO* and *rrnJ*, we suspect that the discrepancy may have resulted from the measurement being done in *E. coli* rather than in *B. subtilis.* Another possibility is that the activity of the P1 promoter of *rrnB* is very low and Deneer and Spiegelman's data could not show it was growth-rate regulated, just as the situation we faced that the activities of the *rrnD* and *rrnB* promoters were too low to determine whether they are stringent-regulated.

The differential regulation of the *B. subtilis rrn* P1 and P2 promoters could have resulted from the sequence heterogeneities around the core promoter regions. For example, we found that the high AT/GC ratio at the UP element region, a hallmark of strong promoters (Ross et al., 1994), is more pronounced in P2 than in P1 for both *rrnO* and *rrnJ* promoters (Table 5). We also noticed that some of the nucleotides flanking the -10, -35 consensus, and discriminators sequences seem to be P1- or P2-specific. For example, preceding -10 and -35

of the P1 promoters are CC(or AC) and GG, respectively, while those of the P2 promoters are TA and GT, respectively. The relative more GC base-pairings in P1 promoters can in fact add more difficulties to melting step in the transcription initiation and thus reduce P1 promoter activity. Beside the unique positioned nucleotides mentioned above, the sequence variations within the -35 and -10 regions of the *B. subtilis rrn* promoters are also apparent, which, as studies in *E. coli* had shown, is essential for growth-rate regulation (Gaal et al., 1989; Dickson et al., 1989; Gourse et al., 1986). We are currently conducting mutation studies on the discriminator region of the *B. subtilis rrnO*-P1 promoter.

The high AT ratio and stretches of uninterrupted A residues are the characteristics of the UAS and UP element regions of rRNA genes (Zacharias et al., 1992; Ross et al., 1994; Federick et al., 1995). The upstream regions of P1 or P2 in *B. subtilis rrnO* and *rrnJ* fit these characteristics (Table 5 and Fig. 20). We found that *rrnO*-UAS is capable of forming complexes with protein(s) in the protein extract preparations isolated from *B. subtilis* strain IS58. The most interesting finding is that this binding property is most prominent during early log phase of bacterial growth, which resembles the expression pattern of *E. coli* Fis protein, a protein transiently expressed during the early log phase. However, the only two prominent *B. subtilis* proteins which could be recognized by anti-Fis serum in Western blot analysis are present at all stages of vegetative growth. We therefore conclude that another as yet uncharacterized protein rather than the two with estimated molecular weight of 45 kd and 65 kd may be responsible for the band shifting in the gel-shift experiments.

We examined the *in vitro* transcription activities of a collection of 9 *rrn* promoters. The data we obtained *in vitro* are generally consistent with our *in vivo* results. They are summarized in Table 3. The most significant finding in the *in vitro* transcription study is the following: 1) the P2 promoter is stronger than P1 as was observed *in vivo*. 2) the P2/P1 ratio increases as NaCl concentration increases, mainly because P1 activity drops precipitously as NaCl concentration raises. P2 promoter remains active both at low and high NaCl concentrations. Transcription initiation is a multistep process, and changes in NaCl concentration may have different effect on each of them. For example, Wellington and Spiegelman (1993) showed that the interaction between *E. coli* RNAP and the P1 and P2 promoters of *B. subtilis rrnB* operon proceeded in three steps and each of which had different sensitivity to heparin. A higher NaCl concentration (e.g. 150 mM) may make a promoter or an RNAP-promoter complex more rigid and less capable of converting from one structure to the other necessary for efficient transcription. Thus, the binding of RNAP to a promoter which is measured by Kd and the transition of the complex from PR_C(closed RNAP-promoter complex) to PR_o (open RNAP-promoter

$$\overset{Kd}{\longleftrightarrow} \quad \overset{Kf}{\longleftrightarrow} \quad \longrightarrow$$

$$R + P \longleftrightarrow PR_C \longleftrightarrow PR_o \longrightarrow RNA$$

complex), which is measured by Kf, may be positively or adversely influenced by higher NaCl concentrations depending on individual promoters (also see review by Perez-Martin et al., 1994). In a recent study on the promoter of the flagellar regulon of *B. subtilis*, Frederick et al. (1995) examined the effect of UP element in promoter competition experiments. It seems that, under less

favorable transcription conditions such as low concentration of RNAP or high concentration of NaCl the activation effect of the UP element was much stronger. For example, when the +UP and -UP templates compete for RNAP, synthesis from the -UP template decreases more than 10-fold, while synthesis from the +UP template is unaffected. Similarly, the +UP template is 19-fold more active than the -UP template at 160 mM NaCl but only 2.4-fold at 20 mM NaCl, presumably because more rapid binding of RNAP to the promoter at low salt condition. These results seem consistent with the hypothesis that the UP element increases the initial binding of RNAP to the promoter (Federick et al, 1995).

The activation by UAS of *in vitro* promoter activity with or without involvement of regulatory proteins had been demonstrated in many experiments using *rrn*, *trn*, and *argT* promoters on short DNA templates (Bosch et al., 1990; Ross et al., 1990; Hsu et al., 1991). The effect of this activation was typically about 5 to 20-fold over the activity of core promoters in various comparisons, and the location of the elements for this activation is between the -35 and -150. Further upstream sequence (-150 to -500) has only marginal effect on activation (less than 10%). Our separated promoter fragments *rrnO*-P1, *rrnO*-P2, and *rrnO*-(-UAS)-P1P2 all contained portion of the sequences upstream of the -35 region (about 120 bp for P1 and about 40 bp for P2), and their activities we observed are not likely the activity of the core promoters because they contained part of UAS region. Therefore, it is conceivable that the enhancement of the entire UAS to the core *rrn* promoters is likely to be higher than the 2-4 folds (in BD170) as we observed in Fig. 10. It should also be

pointed out that we have only observed the enhancing activity of the UAS regions in the *in vivo* expression studies. In the *in vitro* transcription assays, however, UAS does not seem to enhance the activities of either the P1 or the P2 promoters. It also had no effect on the relative strength of the P1 and P2 promoters (Figs. 28 and 29). This could be the indication that transcription activating protein factors are involved in *in vivo rrn* transcription.

The *in vivo* and *in vitro* studies on the P1 and P2 promoters of the *B. subtilis rrnO* and *rrnJ* operons presented in this thesis consistently demonstrated that the P2 promoters are relatively stronger than their corresponding P1 promoters. It is also clear that the P1 and P2 promoters in the *B. subtilis rrnO* and *rrnJ* operons are similarly growth-rate regulated. The P2 promoters are more sensitive than P1 promoters in response to stringent condition induced by amino acid starvation, and only P2 promoters are responsive to stringent condition induced by glucose starvation.

SUMMARY

1. Separated P2 promoters of *rrnO* and *rrnJ* are more active than their corresponding P1 promoters. Both P1 and P2 promoters are growth-rate regulated.

2. Separated P1 and P2 promoters of *rrnO* and *rrnJ* are all subject to stringent condition caused by amino acid starvation.

3. Separated P2 promoters of *rrnO* and *rrnJ* are subject to stringent condition caused by glucose starvation; separated P1 promoters of *rrnO* and *rrnJ* are not.

4. In their natural P1-P2 configuration, P2/P1 ratio is 10-12 for *rrnO* and *rrnJ*, respectively, and both are growth-rates regulated. The stronger P2 promoters exert inhibitory effect on the weaker P1 promoters.

5. In their natural P1-P2 configuration, both P1 and P2 promoters of *rrnO* and *rrnJ* respond to stringent condition induced by amino acid starvation; only the P2 promoters respond to stringent condition induced by glucose starvation.

6. In *in vitro* transcription assay, P2 promoters are more active than P1 in both *rrnO* and *rrnJ* promoters, and both in solitary and in P1-P2 dual configuration, although the P2/P1 ratio is greater when the two promoters are in their natural configuration.

7. P1 is a NaCl-sensitive promoter and its activity declines rapidly when NaCl concentration is raised; P2 is a NaCl-insensitive promoter whose activity remains high both at low and high NaCl concentrations.

8. The UAS regions in *rrnO* and *rrnJ* contain *cis* activating activity. This enhancing activity was observed in the *in vivo* expression studies. In the *in vitro* transcription assays, however, UAS does not seem to enhance the activities of either the P1 or the P2 promoters. This could be the indication that transcription activating protein factors are involved in *in vivo rrn* transcription.

9. *E. coli* RNAP is capable to utilize *B. subtilis rrn* promoter templates similar to *B. subtilis* RNAP, although it may be less capable than *B. subtilis* RNAP to distinguish the different intrinsic strength of the *B. subtilis rrnO* P1 and P2 promoters.

10. A binding activity for *rrnO*-UAS was found most prominent at the early exponential growth phase. This *B. subtilis* protein is capable of interacting with the UAS region of *rrnO* at two separate sites and forms stable complexes. However, neither the 45 kd nor the 64 kd protein which are recognized by anti-Fis serum are responsible for the observed activities.

TABLE 1. Promoter regions of *B. subtilis* and *E. coli rrn* operons

	P1				P2				m16S
	-35 [a]		-10		-35		-10		+1
B. subtilis[b]									
rrnO	TTTACA	16	TATAAT	69	TTGACC	17	TACTAT	179	TTTAT
rrnA	TTGACT	17	TATTCT	50	TTGACA	17	TATCAT	117	TTTAT
rrnJ	TTGCAC	17	TATATT	57	TTGACT	17	TATAAT	118	TTTAT
rrnH		----------		-----	TTGACT	17	TATACT	122	TTTAT
rrnG		----------			TTGACT	17	TATACT	123	TTTAT
rrnW		----------			TTGACT	17	TATACT	122	TTTAT
rrnD	TTGACT	17	TATATT	68	TTGCAC	17	TATATT	182	TTTAT
rrnB	TTGCAA	17	TATATT	63	TTGACA	17	TATATT	178	TTTAT
E. coli[c]									
rrnA	TTGTCA	16	TATAATG	90	TTGACT	16	TATTATG	202	AAAT
rrnB	TTGTCA	16	TATAATG	90	TTGACT	16	TATTATG	203	AAAT
rrnC	TTGTCA	16	TATAATG	89	TTGACT	16	TATTATG	203	AAAT
rrnG	TTGTCA	16	TATAATG	89	TTGACT	16	TATTATG	204	AAAT
rrnD	TTGTGC	16	TATAATG	80	TTGACT	16	TAATATA	205	AAAT
rrnE	TTGCGC	16	TATAATG	81	TTGACT	16	TAATATA	203	AAAT
rrnH	TTGTCT	16	TATAATG	81	TTGACT	16	TAATATA	204	AAAT

a) Numbers in between the consensus sequences indicate the number of nucleotides between the -35 and -10 consensus sequences or the starting site of the 16S rRNA gene shown;

b) References for *B. subtilis* sequences are Ogasawara et al. (1983a); Stewart and Bott (1983); Green et al. (1985); Green and Vold (1992); Wawrousek and Hansen (1983); Jarvis et al. (1988) and Widom, 1988.

c) *E. coli* data were taken from Jinks-Robertson and Nomura, 1987.

TABLE 2. Primers used for PCR reactions

Primer name	Sequence*	Restriction Site	Purpose**
R15-71	GGGGGATCCGCTCGACTTGCATGTAT	BamHI	rrnJ-P2,rrnJ-P1P2,rrnO-P2
R15-72	GGGAAGCTTGCCGCTAAACAAGGCG	Hind III	rrnO-P1P2,rrnO-UAS-P1P2 rrnJ-P2
R15-73	GGGGAATTCCCCTTCTATTCGCGAT	EcoRI	rrnJ-P1,rrnJ-P1P2
R15-74	GGGAAGCTTCGCCTTGTTTAGCGGC	HindIII	rrnJ-P1
R15-77	CCGGATCCTGCAGACACAAGCATGACC	BamHI, PstI	rrnO-P2
R15-78	CCGGATCCTAGTCATAATGGTCATGC	BamHI	rrnO-P1, rrnO-UAS-P1
R15-79	GGGAAGCTTCTGCAGGTGTGCGTCTCAT	HindIII, PstI	rrnO-P1, rrnO-P1P2
R15-80	CCCGAATCAATGCGGAGCTT	EcoRI	rrnO-UAS,rrnO-UAS-P1
R15-81	CCCAAGCTTGAGTGACACGTCC	HindIII	rrnO-UAS-P1P2 rrnO-UAS

*double underlines indicate regions of primers complementary to target template DNA; single lines indicate the sequences recognized by restriction enzymes.

**some primers are used to generate several different promoter fragments

TABLE 3. Fragments of *rrn* promoters and *rrnO*-UAS created for this study

PCR fragment	Size (bp)	Relative promoter activity			
		in vivo [a]		*in vitro* [b]	
		β-gal	RNA	50 mM	150 mM
rrnO	1900	970	3855	0	0
rrnO-P1	240	550	1178	297	448
rrnO-P2	341	850	2133	2414	4656
rrnO-P1P2	549	n.d.	n.d.	3201	5541
rrnO-UAS-P1P2	1075	n.d.	n.d.	1463	5538
rrnO-UAS	549	—	—	—	—
rrnO-UAS-P1	754	n.d.	n.d.	163	842
rrnJ	1500	1076	1685	0[c]	n.d.
rrnJ-P1	194	290	6010	0[c]	n.d.
rrnJ-P2	269	436	1178	4051[c]	n.d.
rrnJ-P1P2	433	487	n.d.	4110[c]	n.d.
rrnJ-P1(H)P2	455	69	n.d.	0[c]	n.d.
pilV[d]	400	n.d.	n.d.	v.a.[e]	v.a.[e]

a) β-galactosidase activity data were obtained in *B. subtilis* strain SB25 growing in MM1; RNA data were *lacZ* mRNA relative amount;
b) activity of *in vitro* run-off assays.
c) data were obtained at 10 mM NaCl.
d) *E. coli* leucine gene promoter obtained from Dr. John Helmann.
e) very active.

TABLE 4. *Bacillus subtilis* strains and plasmids used in this study

Strain or plasmid	Genotype	Size (Kb)	Source
I. BACTERIAL STRAINS			
IS58	*trpC2, lys-3, relA*$^+$		I. Smith
IS56	*trpC2, lys-3, relA*$^-$		I. Smith
SB25	*trpC2, hisH2*		R. Rudner
BD170	*trpC2, thrA5*		R. Rudner
II. PLASMIDS[1]			
pGEM-Cat3Z	*amp*^{R},*cm*^{R}, *lacZ(P*$^-$*)*	3.2 [2]	Youngman
pDH32	*amp*^{R},*cm*^{R},*amyE,spoVG-lacZ(P*$^-$*)*	9.9 [2]	D. Henner
pDG268	*amp*^{R},*cm*^{R},*amyE,spoVG-lacZ(P*$^-$*)*	9.3 [2]	P. Stragier
pPW4	pDH32-(P1-P2)-*rrnO*	1.9	C. Stewart
pAWR118	pDG268-(P1-P2)-*rrnJ*	1.5	A-M.White
pAWR116	pDG268-(P1-P2)-*rrnD*	2.2	A-M.White
pAWR123	pDG268-(P1-P2)-*rrnB*	1.8	A-M.White
pPW810	pDH32-(P1)-*veg*	0.48	C. Stewart
pLR105	pDG268-(P2)-*rrnJ*	0.269	D. Liu
pLR210	pDG268-(P1)-*rrnJ*	0.194	D. Liu
pLR203	pDG268-(P1-P2)-*rrnJ*	0.441	D. Liu
pLR201	pDG268-(P1-*Hind*III-P2)-*rrnJ*	0.463	D. Liu
pLR501	pDG268-(P1)-*rrnO*	0.341	D. Liu
pLR512	pDG268-(P2)-*rrnO*	0.24	D. Liu
pLR204	pGEM-Cat3Z-(P1)-*rrnJ*	0.194	D. Liu
pLR139	pGEM-Cat3Z-(P2)-*rrnJ*	0.269	D. Liu
pLR519	pGEM-Cat3Z-(UAS)-*rrnO*	0.549	D. Liu

(1) All plasmids were introduced into *B. subtilis* strains at the *amyE* locus.
(2) The sizes of the plasmids.

TABLE 5. The sequences of *B. subtilis rrnO* and *rrnJ* promoter elements

Promoter	Sequence[a]	Regulation[b] SHX	αMG
rrnO-P1	CTGCGCTTTTTTGTGTCATAACCC**TTTACA** GTCATAAAAATTATGG**TATAAT**CATTTCT**GTTGTCT**	+	–
rrnO-P2	AGTAAAAACTTTTTCAAAAAAGTA**TTGACC**TAGTTAACTAAAAATGT**TACTAT**TAAGTA **GTCGCTT**	+	+
rrnJ-P1	CCAGTAGTATTTCTTCAAAAAAAC**TTGCAC**TATTATTTACTAGGTGG**TATATT**ATTATT**CGTTGCCG**	+	–
rrnJ-P2	AAAAAAGAACTTCAAAAAAAGTTA**TTGACT**TCACTGAGTCAAGGAGT**TATAAT**AATAAA**GACGTACC**	+	+
	UP element **-35** **-10** **Discriminator**		

[a] The *B. subtilis* promoter sequences of *rrnO* and *rrnJ* are from Ogasawara et. al. (1983a) and Widom (1988), respectively.

[b] The ability (+) or inability (-) of a promoter to be regulated under nutritional stress conditions SH or αMG is indicated.

Figure 1. Chromosomal map of *B. subtilis* 168T

Chromosomal map of *B. subtilis* 168T with the positions of all 10 rRNA genes and all the reported tRNA gene clusters associated with them as summarized by Rudner et al. (1993). The number of tRNA in each cluster is indicated in the parenthesis. The map is based on the genetic mapping reported by Jarvis et al. (1988). The basic reference for the known loci, including *amyE* and *thrC*, is taken from the mapping kit strains of Dedonder et al. (1977).

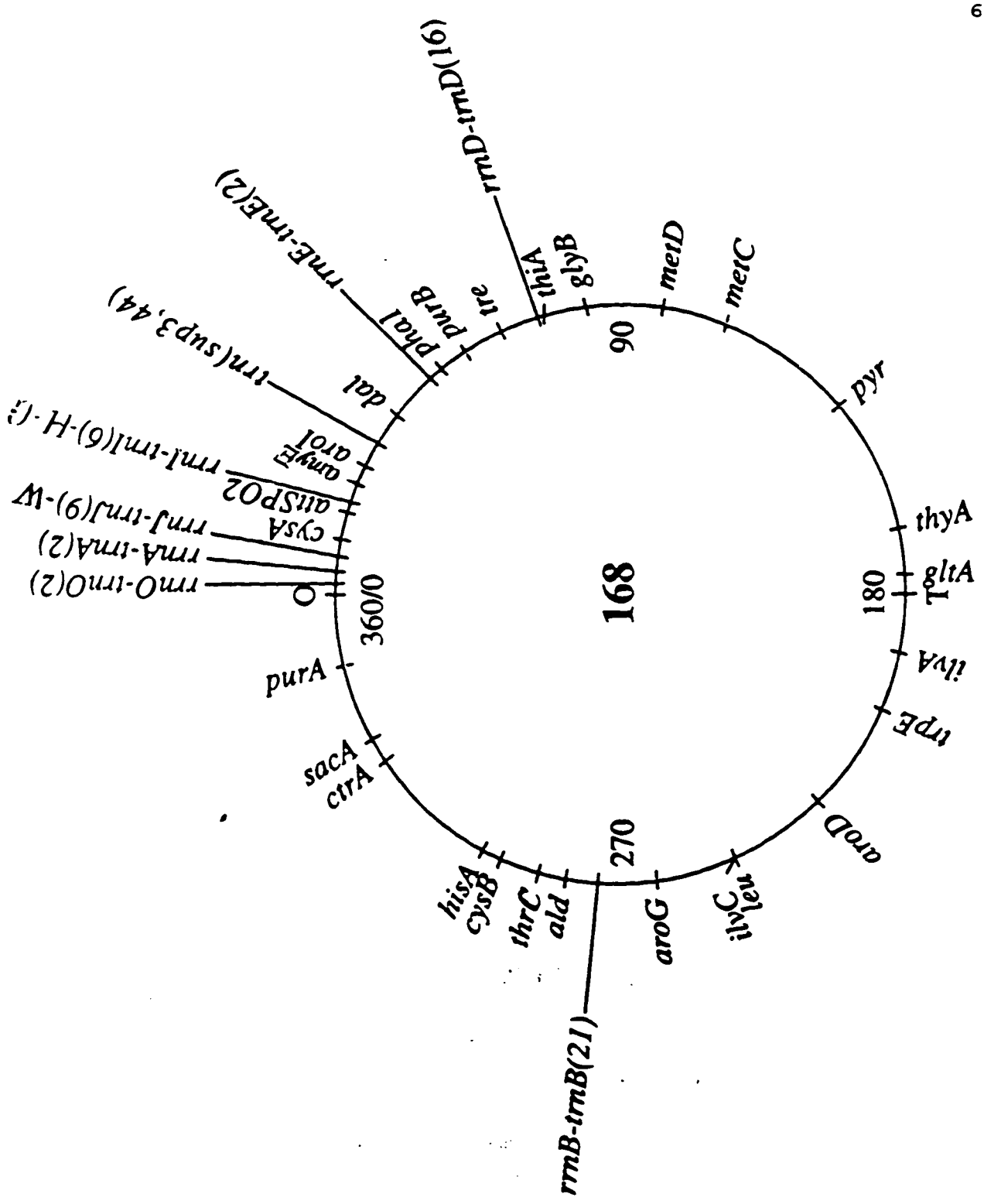
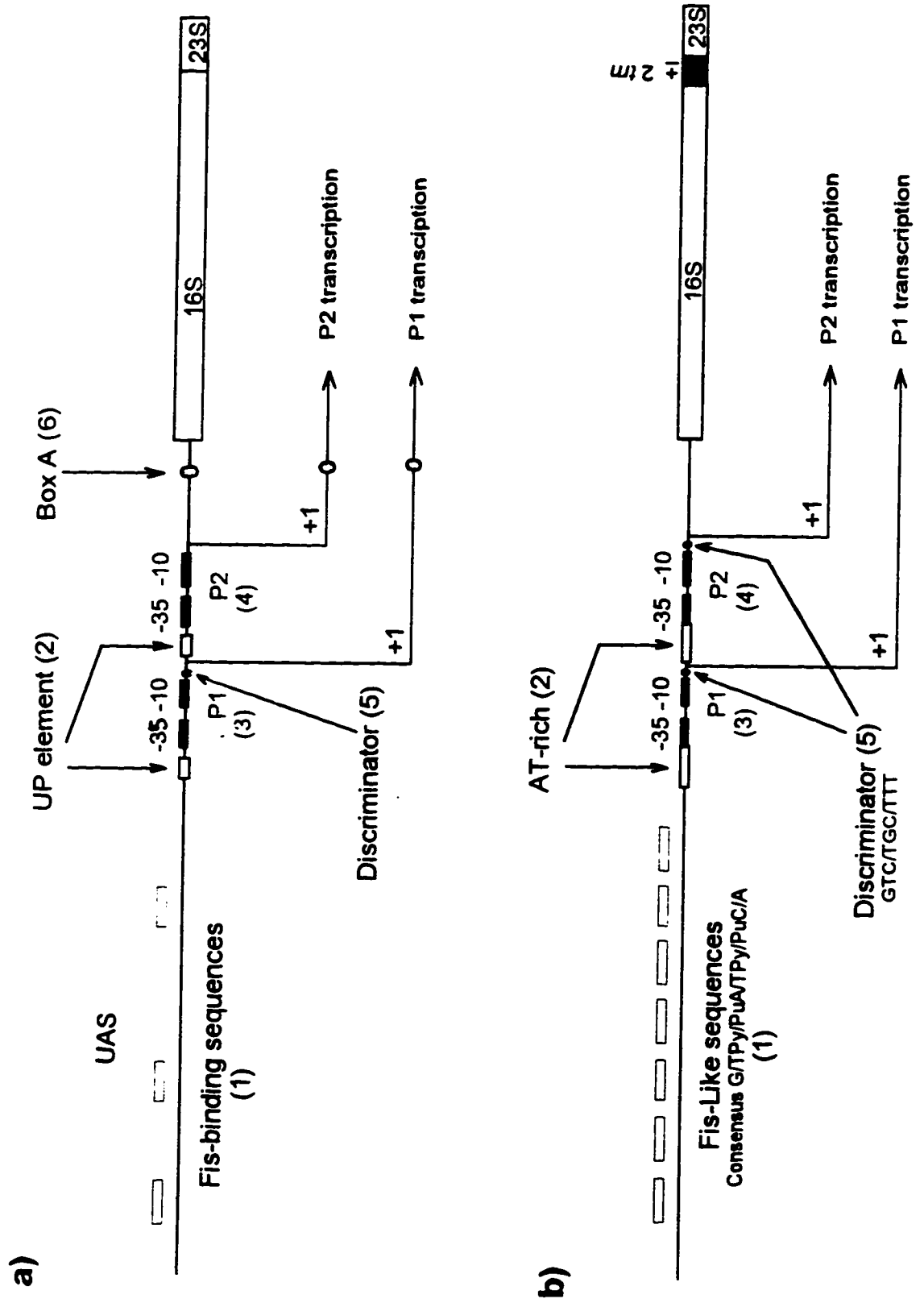


Figure 2. Transcription regulation elements identified in the *E. coli* and *B. subtilis* *rrn* promoter regions

a) *E. coli rrn* operons: six elements were found important to *rrn* promoter regulation: 1) three Fis binding sites (*rrnB*) are located in the UAS region (Gourse et al., 1986); 2) UP element which interacts with the α subunit of RNAP is centered at -55 (Ross et al., 1994); 3) upstream P1 promoter; 4) downstream promoter P2; 5) discriminator sequence GCGC downstream of P1, which is believed to be the sequence determinant for stringent control regulation; and 6) anti-terminator, also called BoxA, is required for antitermination, allowing RNAP to read through the long, non-translating-rRNA gene (Squires et al., 1993).

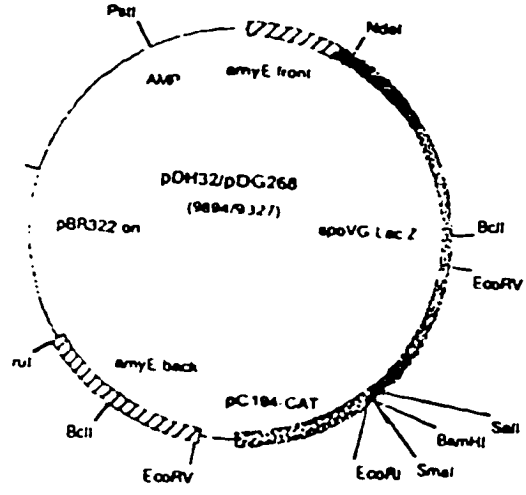
b) *B. subtilis rrn* operons: Fis consensus sequences were found in the UAS region and they could be the candidates of the target of a Fis-like protein in *B. subtilis*. Both P1 and P2 promoters seem to have their own discriminators located between the -10 and +1. AT-rich sequences are found preceding the -35 consensus sequences of both P1 and P2 (see text). Only *rrnO* and *rrnA* contain two tRNA gene in the spacer region of 16S and 23S rRNA genes, other *rrn* operons cluster with tRNA genes at the end of the 23S rRNA gene.



Figures 3. Physical maps of the plasmids used in this study

a) pDH32/pDG268: single-copy integration vectors for *B. subtilis* that allow a targeted integration of a single copy of a *lacZ* fusion into the *amyE* gene. The essential features of the plasmids are that they contain a promoterless *lacZ-spoVG* with a *B. subtilis* RBS (Ribosomal Binding Site) and a Cm^R determinants, flanked by larger regions of *amyE*. The *EcoRI-HindIII-BamHI* polylinker is the region promoters are inserted. Four *rrn* intact promoter fragments (*rrnO, rrnJ, rrnB, rrnD*) and seven separated PCR-produced promoter fragments (P1, P2, and P1P2 of *rrnO* or *rrnJ*) were joined to the *spoVG-lacZ* reporter gene in pDH32/pDG268. b) pGEM-Cat3Z, the plasmid used for double strands DNA sequencing. It carries Ap^R and Cm^R determinants. The sizes of all the plasmids are labeled.

a)



b)

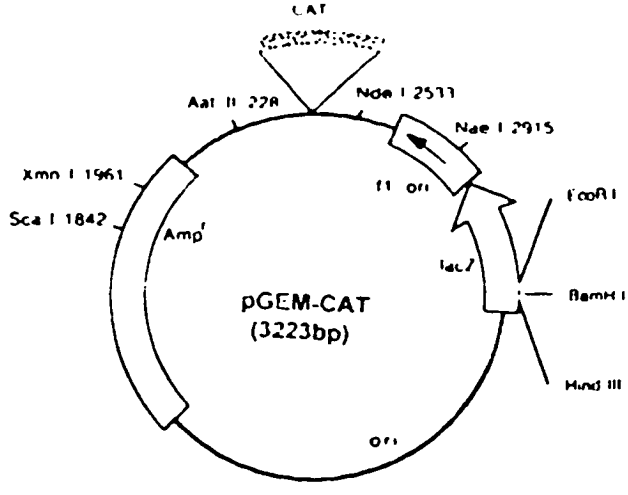
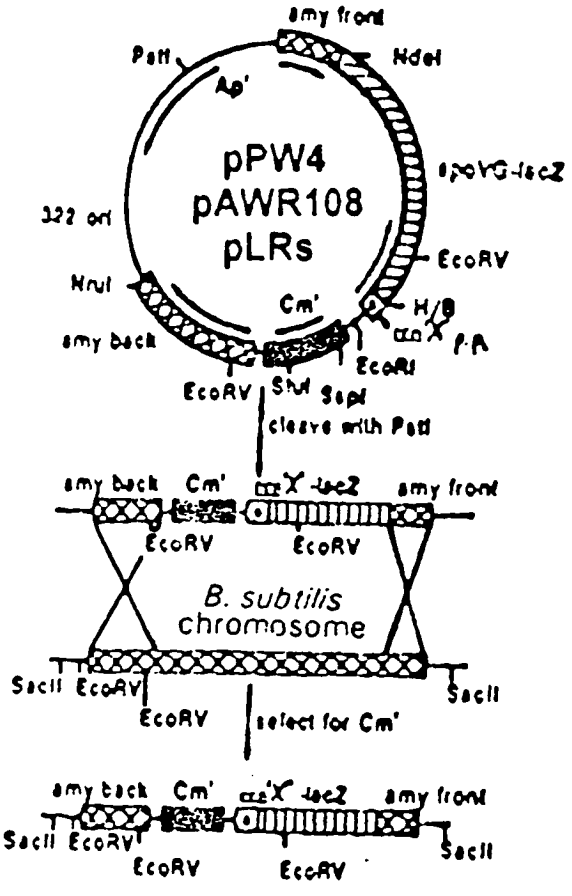


Fig. 4. Illustration of the integration of pDH32/pDG268 and its derivatives at the *amyE* locus of *B. subtilis*

a) replacement integration by double cross-over replacing the middle part of the *amyE* gene on the chromosome and yielding an *amyE*$^-$ strain. b) insertion integration by a single cross-over event (Campbell like) yielding an *amyE*$^+$ strain. A single cross-over may take place at the "*amyE* front" or "*amyE* back" regions, producing bands with slightly different sizes in Southern blot analysis (see Fig. 5).

a) replacement integration



b) insertion integration

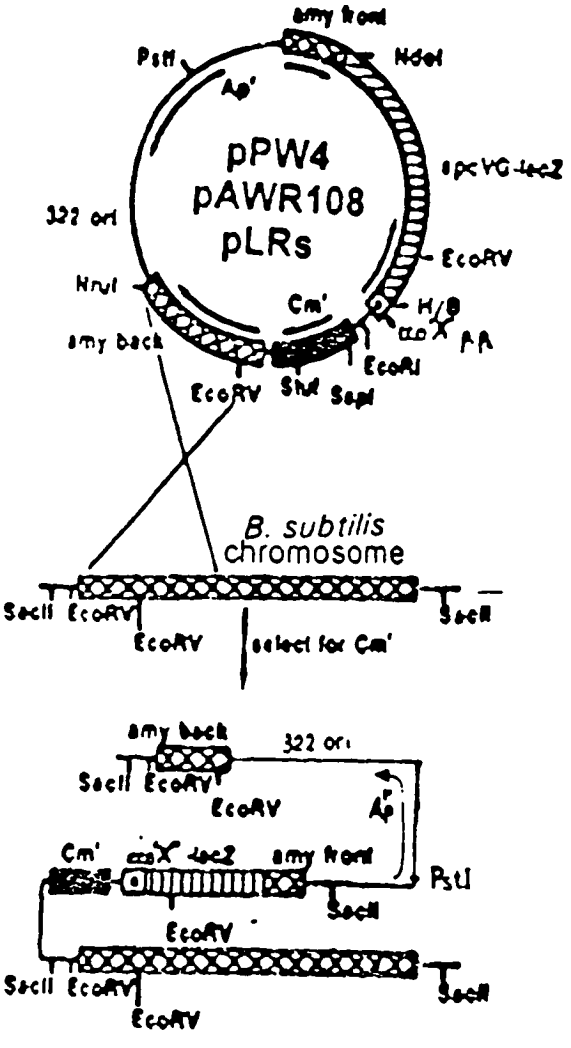


Fig. 5. Southern blot analysis: verification of integrations at the *amyE* locus

B. subtilis was transformed with either PstI-linearized or circular plasmids and the integrants were selected by their Cm^R determinant. Different recombinations were analyzed by Southern hybridizations of EcoRV-restricted chromosomal DNA from Cm^RamyE$^-$ or Cm^RamyE+ clones (this was done by Ann-Marie White and Barbara Studamire). The radioactive probe was a 1318 bp DNA fragment from the *amyE* back region obtained from an EcoRV-NruI double digestion of pDG268 (Fig. 3). The predicted size and number of hybridization bands for different types of integration are: a) double cross-over, or, *amyE*$^-$: 1.4 kb; b) single cross-over at *amyE* front: 1.4 and 6.1 kb; c) single cross-over at *amyE* back: 1.4 and 6.3 kb; d) integration at an *rrn* locus: 1.4 and 1.4 kb (Dr. Rudner unpublished results). Lane 1, λ-DNA digested with HindIII; Lane 2, *rrnD-amyE+*; Lane 3, *rrnO-amyE+*; Lane 4, *rrnO-amyE+*; Lane 5, *rrnD-amyE*$^-$; Lane 6, *rrnD-amyE+*; Lane 7, *rrnO-amyE*$^-$, Lane 8, *rrnO-amyE*$^-$ (Southern blot data were done by Ann-Marie White and Barbara Studamire).

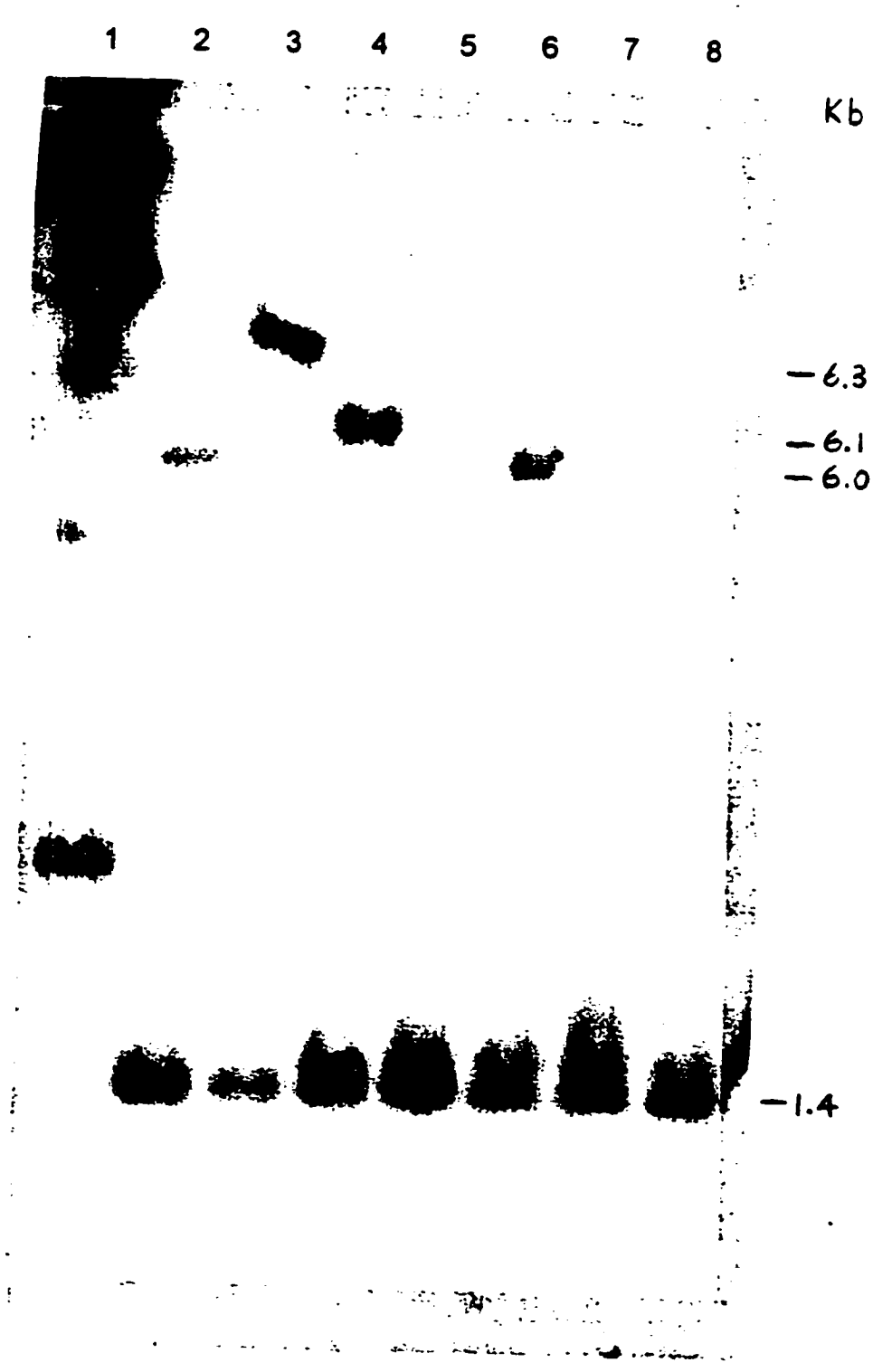
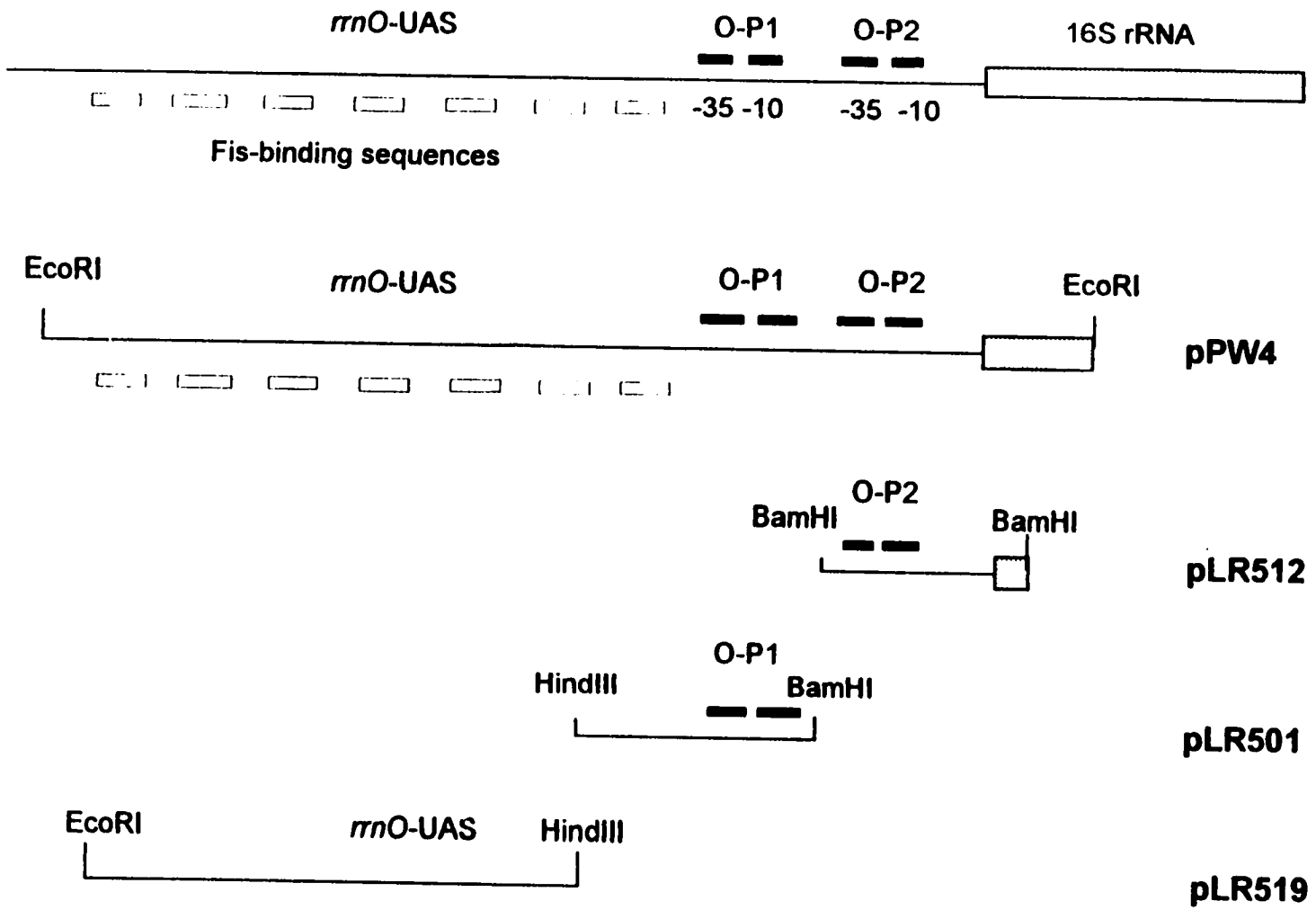


Fig. 6. Schematic diagram of the separation of P1, P2 and the UAS of the *B. subtilis rrnO* operon

DNA fragments of *rrnO*-P1, *rrnO*-P2, and *rrnO*-UAS were created by PCR using the primers described in Table 2 and the conditions for PCR reactions are described in Materials and Methods. The restriction sites at the ends of the PCR fragments were created by designing them on PCR primers. The *in vivo* expression activities were determined by both β-galactosidase assay by the method of Miller (1972) and by RNA dot blot analysis (Samarrai et al., 1995). The *in vivo* P2/P1 ratio for the solitary promoters was 1.6 (β-galactosidase activity) and 2.3 (relative RNA). For the intact *rrnO* (pPW4) the P2/P1 ratio was 10 in a primer extension analysis (see text).

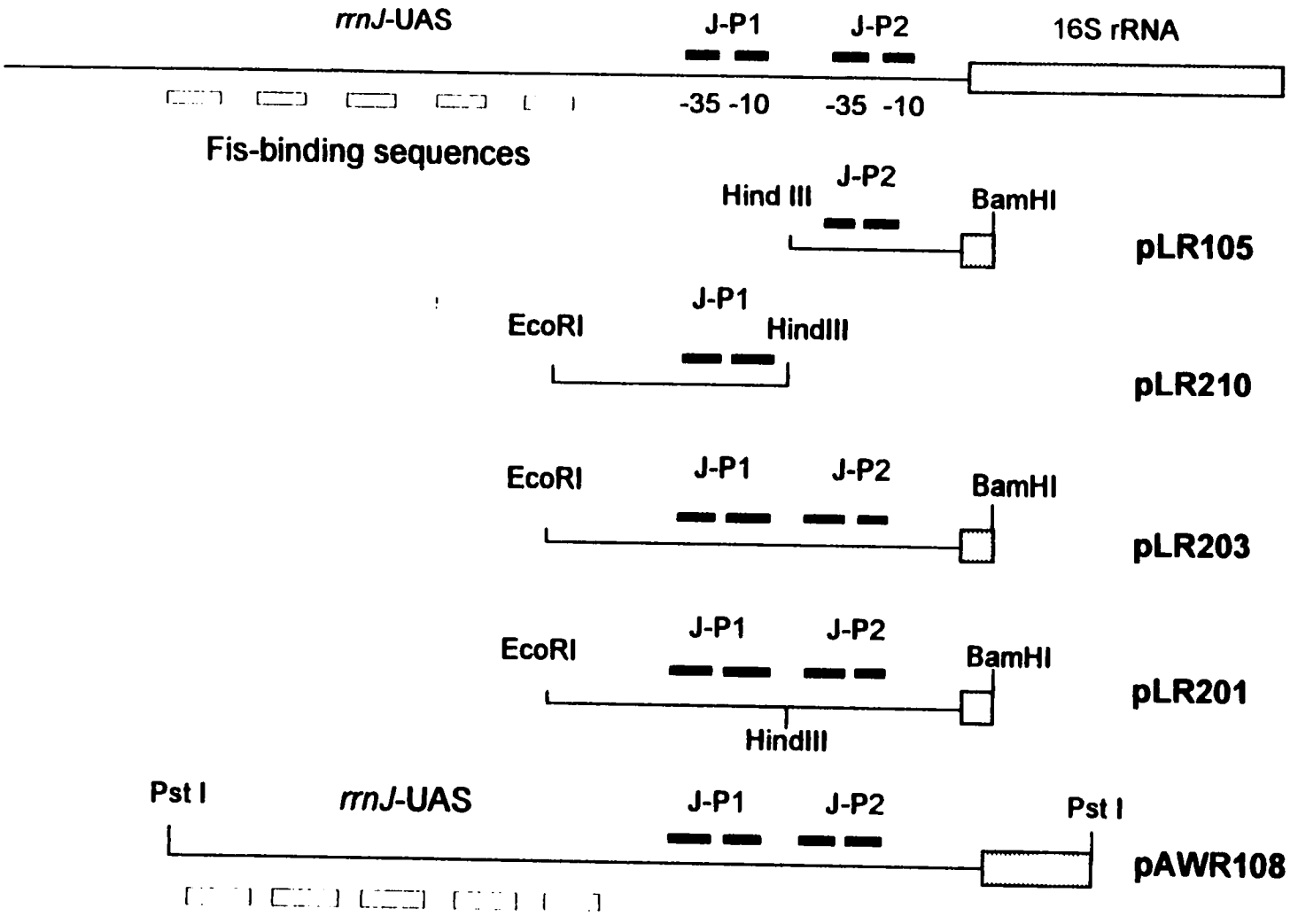


	In vivo activity (% of wild type)	
	β-gal	RNA[a]
pPW4	100	100[b]
pLR512	52	60
pLR501	31	26
pLR519	n.a.[c]	n.a.[c]

a) Data from RNA dot blot; b) Primer extention ratio of P2/P1 = 10.
c) not applicable. Cultures were grown in Spizizen minimum salts-1% glucose

Fig. 7. Schematic diagram of the separation of P1, P2 and P1P2 of the *B. subtilis* *rrnJ* operon

DNA fragments of *rrnJ*-P1, *rrnJ*-P2, and *rrnJ*-P1P2 were created by PCR using the primers described in Table 2 and the conditions for PCR reactions are described in Materials and Methods. The restriction sites at the ends of the PCR fragments were created by designing them on PCR primers. The *rrnJ*-P1(H)P2 was created by joining *rrnJ*-P1 and *rrnJ*-P2 at their HindIII sites. The *in vivo* expression activities were determined by both β-galactosidase assay by the method of Miller (1972) and by RNA dot blot analysis (Samarrai et al., 1996). The P2/P1 ratio for the solitary promoters was 1.7 and 1.6 for β-galactosidase activity and relative RNA amount, respectively. For the intact *rrnJ* (pAWR118) the P2/P1 ratio was 12.5 in a primer extension analysis (see text). *rrnJ*-P1(H)P2 in pLR201 has a 22 bp insertion between P1 and P2 as compared to the natural context in *rrnJ*-P1P2 due to overlaps of the primers used (Table 2). Although both have the same endpoints the activity of *rrnJ*-P1(H)P2 in pLR201 is only 14% of that of *rrnJ*-P1P2.



	In vivo activity (% of wild type)	
	β-gal	RNA[a)]
pLR105	57	63
pLR210	34	39
pLR203	65	n.d.[c)]
pLR201	8	n.d.[c)]
pAWR108	100	100[b)]

a) Data from RNA dot blot; b) Primer extention ratio of P2/P1 = 12;
c) not determined. Cultures were grown in Spizizen minimum salts-1% glucose

Fig. 8. The relative activity and growth regulation of the *rrnO* and *rrnJ* promoters at their resident loci

An *rrn*-lacZ construct containing *E. coli* RBS was inserted downstream of *rrnO* and *rrnJ* promoters in their native chromosomal location in *B. subtilis* strain 168T (Rudner et al., unpublished result). Integrants were grown in MM-Glu and VY media, in which the doubling time was 0.58 and 1.58, respectively. Expressions were measured in β-galactosidase Miller Units (original data were taken from Russell Widom's thesis; Widom, 1988).

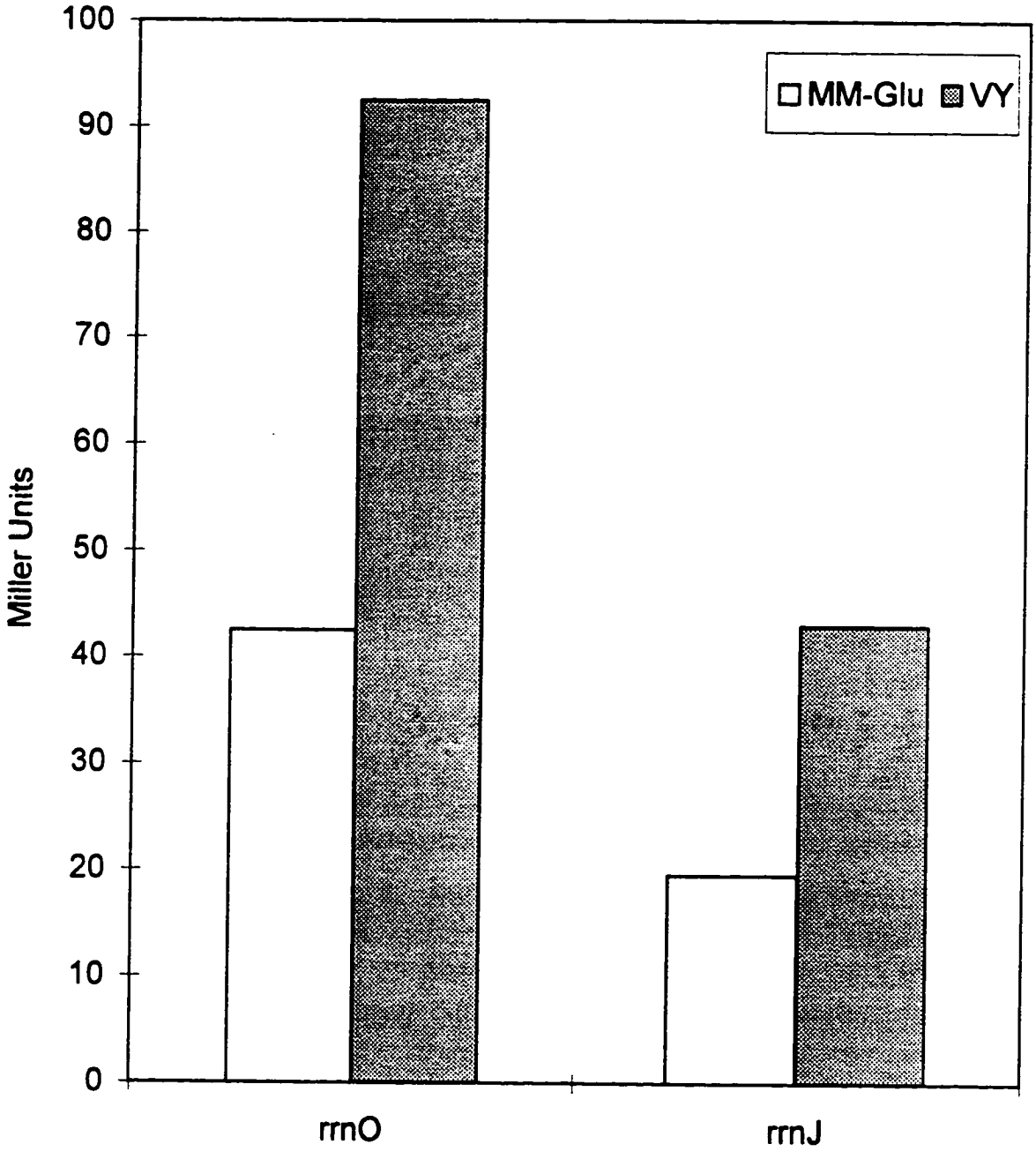


Fig. 9. The relative activity and growth regulation of the *rrnO* and *rrnJ* promoters at *amyE* locus

The *rrn*O promoter cloned in pDH32 (C. Stewart, Table 4) or *rrnJ* promoter in pDG268 (A-M. White, Table 4) and integrated into *B. subtilis* SB25 genome at the *amyE* locus. Integrants were *amyE*$^-$ and they were grown in three different media which support bacterial growth-rates of 0.25, 0.35, and 0.60 doubling per hour. Miller units of β-galactosidase activity were obtained using the procedure (1) described in Materials and Methods. Two of one-ml cell cultures were taken at various Klett readings (92, 176, and 264 for MM-Glu; 59, 117, and 158 for MM-Suc; 70, 100, and 113 for MM-Ac) . The average Miller units for each promoter is the average of the two sets of measurements, each consisting determinations corresponding to the three Klett readings. This measurement was done by Caroline Barangan.

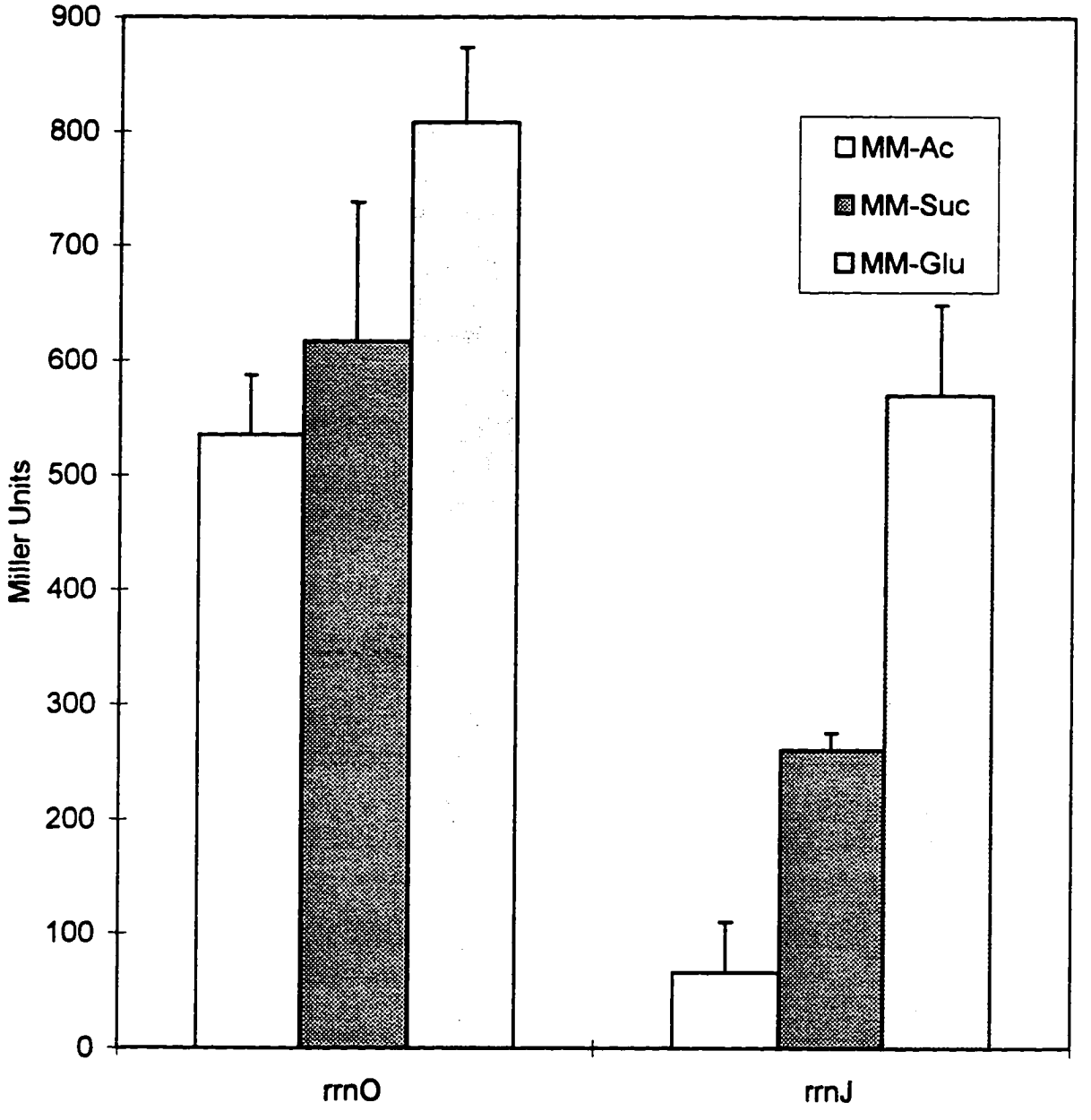


Fig. 10. Expression of *rrnJ* promoters in *B. subtilis* strain BD170 as a function of different growth-rates

The four *rrn*J promoters (*rrnJ*-UAS-P1P2, *rrnJ*-P1P2, *rrnJ*-P1 and *rrnJ*-P2, Table 3) were cloned in pDG268 and integrated at *amyE* locus in *B. subtilis* strain BD170. Integrants were grown in three different media which support growth-rates ranging from 0.25 to 1.35 doubling per hour. Activities are expressed in β-galactosidase Miller units (Materials and Methods). There is a significant drop in *rrnJ*-P1P2 with UAS in VY medium probably due to faster degradation in fast growth (see text). The Miller units for each promoter is the average of the two sets of measurements. This measurement was done by Sharina Mangal.

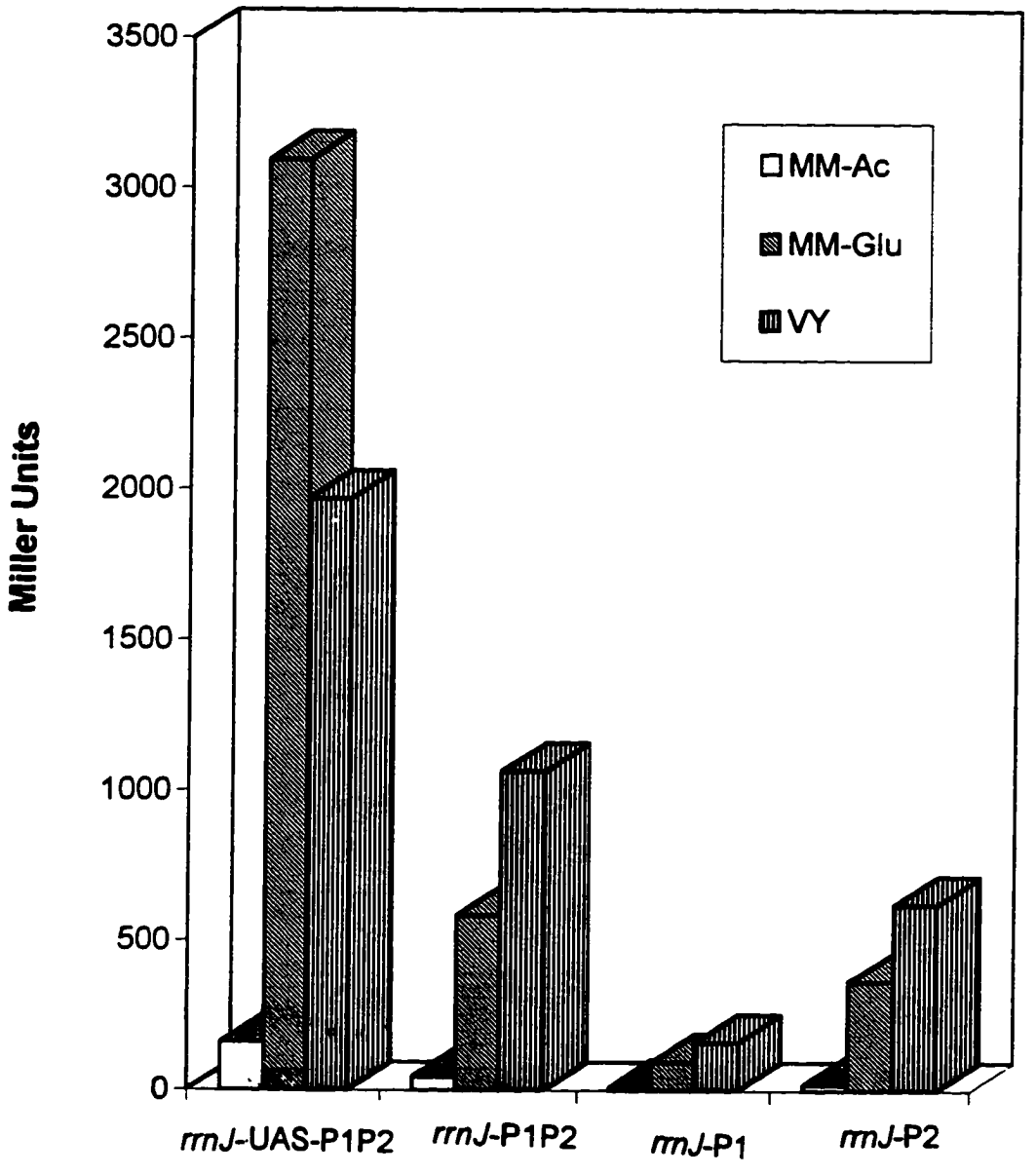


Fig. 11. Expression of *rrnJ* promoters in *B. subtilis* strain SB25 as a function of different growth-rates

The *rrnJ* promoters (*rrnJ*-P1 and *rrnJ*-P2, Table 3) were cloned in pDG268 and integrated at *amyE* locus in *B. subtilis* strain SB25. Integrants were grown in LB and MM-glucose media. Activities are expressed in β-galactosidase Miller units. Miller units were calculated from two to three independent experiments with each of them having two sets of parallel measurements (see Materials and Methods). Samples were taken from cultures at Klett readings as following: 76, 146, and 222 for P1/MM-Glu; 59,119, and 324 for P1/LB; 58, 106, and 199 for P2/MM-Glu; 65, 95, and 285 for P2/LB.

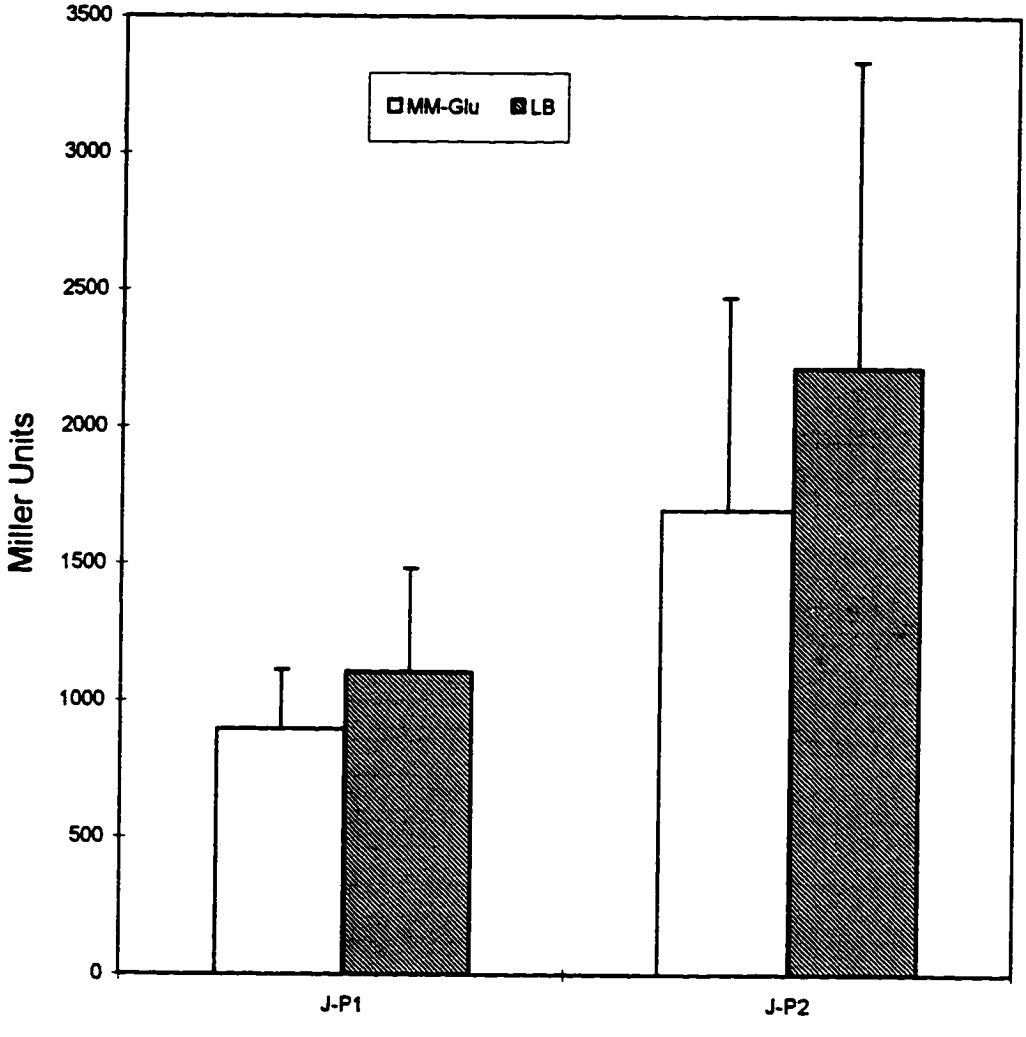


Fig. 12. Expression of *rrnO* promoters in *B. subtilis* strain SB25 as a function of different growth-rates

The *rrnO* promoters (*rrnO*-P1P2 with UAS, *rrnO*-P1 and *rrnO*-P2, Table 3) were cloned in pDG268 and integrated at *amyE* locus in *B. subtilis* strain SB25. Integrants were grown in VY and MM-glucose media in which the growth rates were 1.35 and 0.60 doubling per hour. Activities are expressed in β-galactosidase Miller units. Data were generated from two to three independent experiments with each of them having two sets of parallel measurements (see Materials and Methods). Samples were taken from cultures at Klett readings as following: 86,165 for (+)P1P2/VY; 52,138 for P2/VY; 79,174 for P1/VY; 67 for (+)P1P2/MM; 71 for P1/MM; 70 for P2/MM. The Miller units for each promoter is the average of the two sets of measurements (0.1 ml and 0.2 ml). This measurement was done by Maria Villacs.

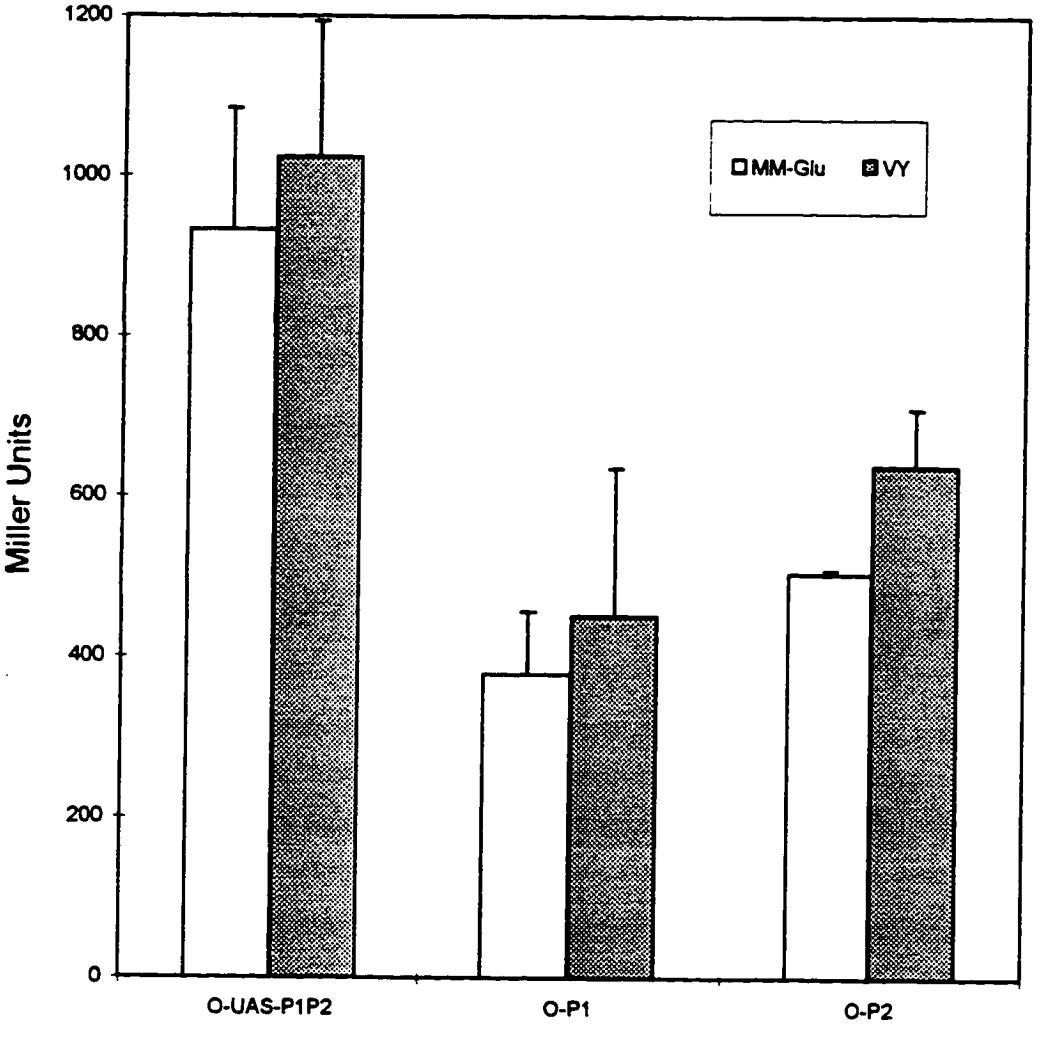


Fig. 13. The intervening sequences between P1 and P2 promoters in pLR203 and pLR201

Only the sequences between the -10 of *rrnJ*-P1 and -35 of *rrnJ*-P2 are shown. Sequence of *rrnJ*-P1P2 was taken from Widom (1988) and that of *rrnJ*-P1(H)P2 was deduced from the primers (Table 2) used to generate it. This 22 bp insert in pLR201 contains a HindIII site and its insertion makes a direct repeat of 21 bp between P1 and P2 promoters. Relative activities *in vivo* and *in vitro* were calculated based on data in Fig. 7 and Fig. 31, respectively, using the activity of *rrnJ*-P1P2 as 100%.

* *rrnJ*-P1(H)P2 did not produce any transcript even at 10 mM NaCl, a very low NaCl concentration (see text).

	Relative activity (%)	
	in vivo	*in vitro*
***rrnJ* P1P2 in pLR203**		
J-P1,-10 J-P2,-35 TATATTAT TATTC GTTGCCGCTA AACAAGGCGA TAACGAAAAA AAGAACTTCA AAAAAAGTTA TTGACT	100	100
***rrnJ* P1(H)P2 in pLR201**		
J-P1,-10 J-P2,-35 TATATTAT TATTC GTTGCCGCTA AACAAGGCGA TAACGAAAAA AAGAACTTCA AAAAAAGTTA TTGACT	14	0*
AAGC TTGCCGCTA AACAAGGCG		

* Both P1 and P2 transcripts are not detectable at 50 mM NaCl.

Fig 14. The effect of α-methyl glucoside (αMG) on the expression of *B. subtilis* *rrnO* promoters

The effect of α-methyl glucoside (αMG) on the expression of *rrnO*-P1P2, *rrnO*-P1, and *rrnO*-P2, *lacZ* fusions (pPW4, pLR501, pLR512) in IS58 and IS56 strains. Bacteria were grown in MM-Glu medium and αMG was added to the cultures when the Klett reached 80 to 85. Samples were taken at 0 and 90 minutes after addition of 1% αMG to the cultures. The levels of *lacZ* mRNA in total RNA were determine by hybridization to probe specific for *lacZ* mRNA. The probe was labeled with the random primer extension kit, using α-^{32}P-dCTP (Samarrai et al., 1996).

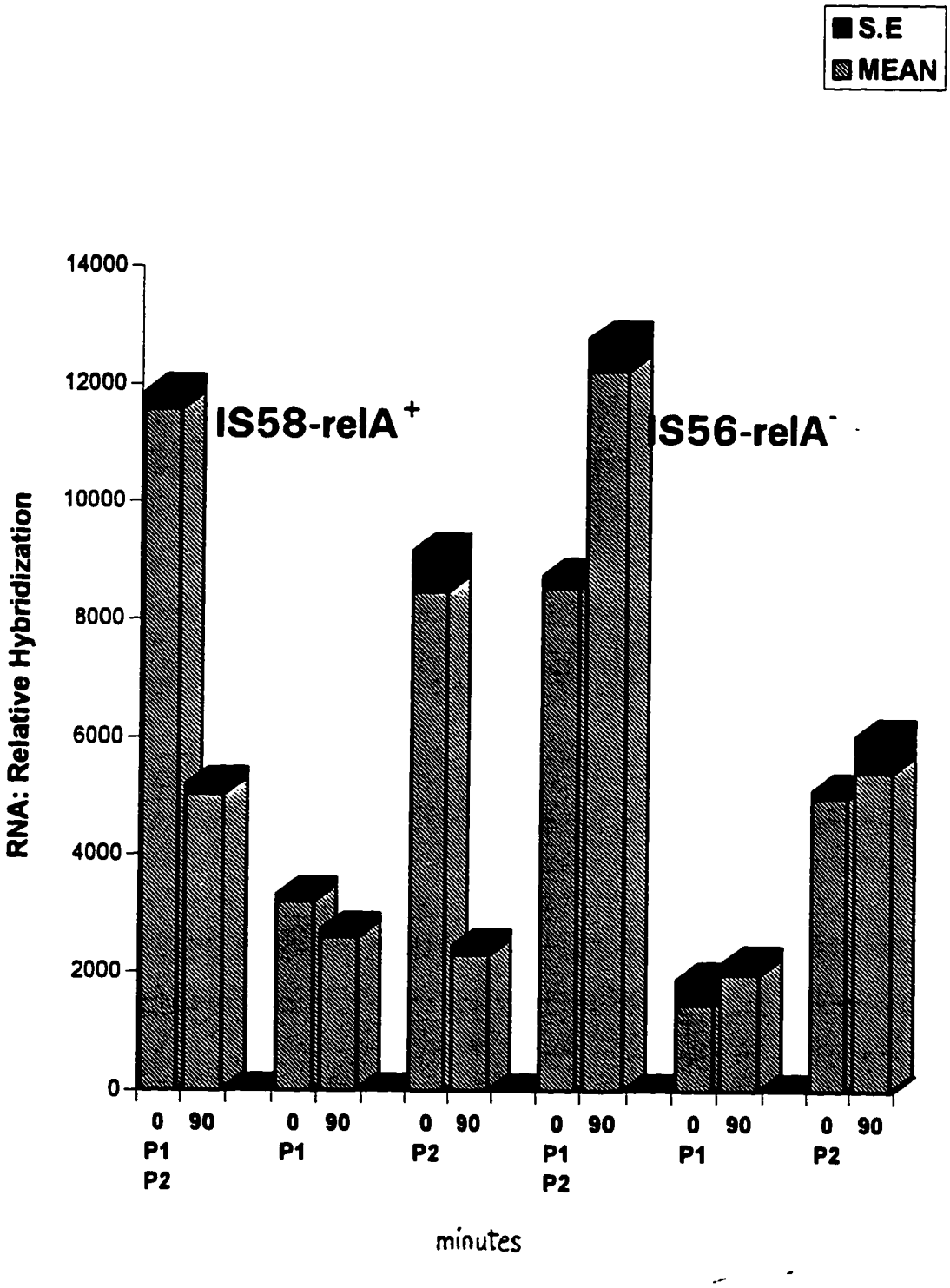


Fig. 15. The effect of α-methyl glucoside (αMG) on the expression of *rrnJ* promoters

The effect of α-methyl glucoside (αMG) on the expression of *rrnJ*-P1P2, *rrnJ*-P1, and *rrnJ*-P2, *lacZ* fusions (pAWR108, pLR210, pLR105) in IS58 and IS56 strains. Bacteria were grown in MM-Glu medium and αMG was added to the cultures when the Klett reached 80 to 85. Samples were taken at 0 and 90 minutes after addition of 1% αMG to the cultures. The levels of *lacZ* mRNA in total RNA were determine by hybridization to probe specific for *lacZ* mRNA. The probe was labeled with the random primer extension kit, using α-^{32}P-dCTP (Samarrai et al., 1996).

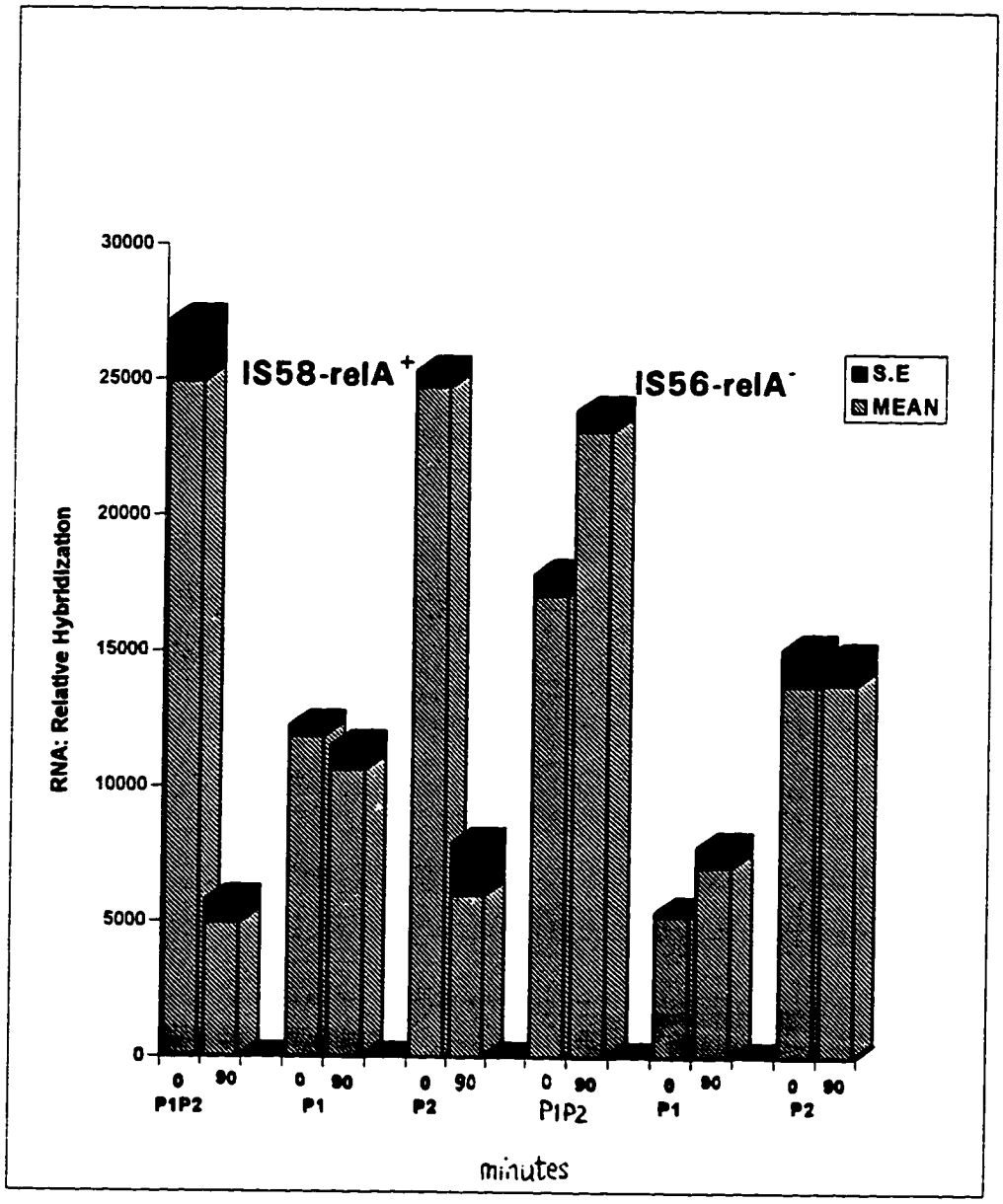


Fig. 16. The effect of α-methyl glucoside (αMG) on the expression of *B. subtilis rrn* promoters

The effect of α-methyl glucoside (αMG) on the expression of *rrnO*-P1P2, *rrnJ*-P1P2, *rrnB*-P1P2, *rrnD*-P1P2, and *veg* (control), *lacZ* fusions (in pPW4, pAWR108, pAWR123, and pAWR116, and pPW810, respectively) in IS58 and IS56 strains. Bacteria were grown in MM-Glu medium and αMG was added to the cultures when the Klett reached 80 to 85. Samples were taken at 0 and 90 minutes after addition of 1% αMG to the cultures. The levels of *lacZ* mRNA in total RNA were determine by hybridization to probe specific for *lacZ* mRNA. The probe was labeled with the random primer extension kit, using α-^{32}P-dCTP (Samarrai et al., 1996).

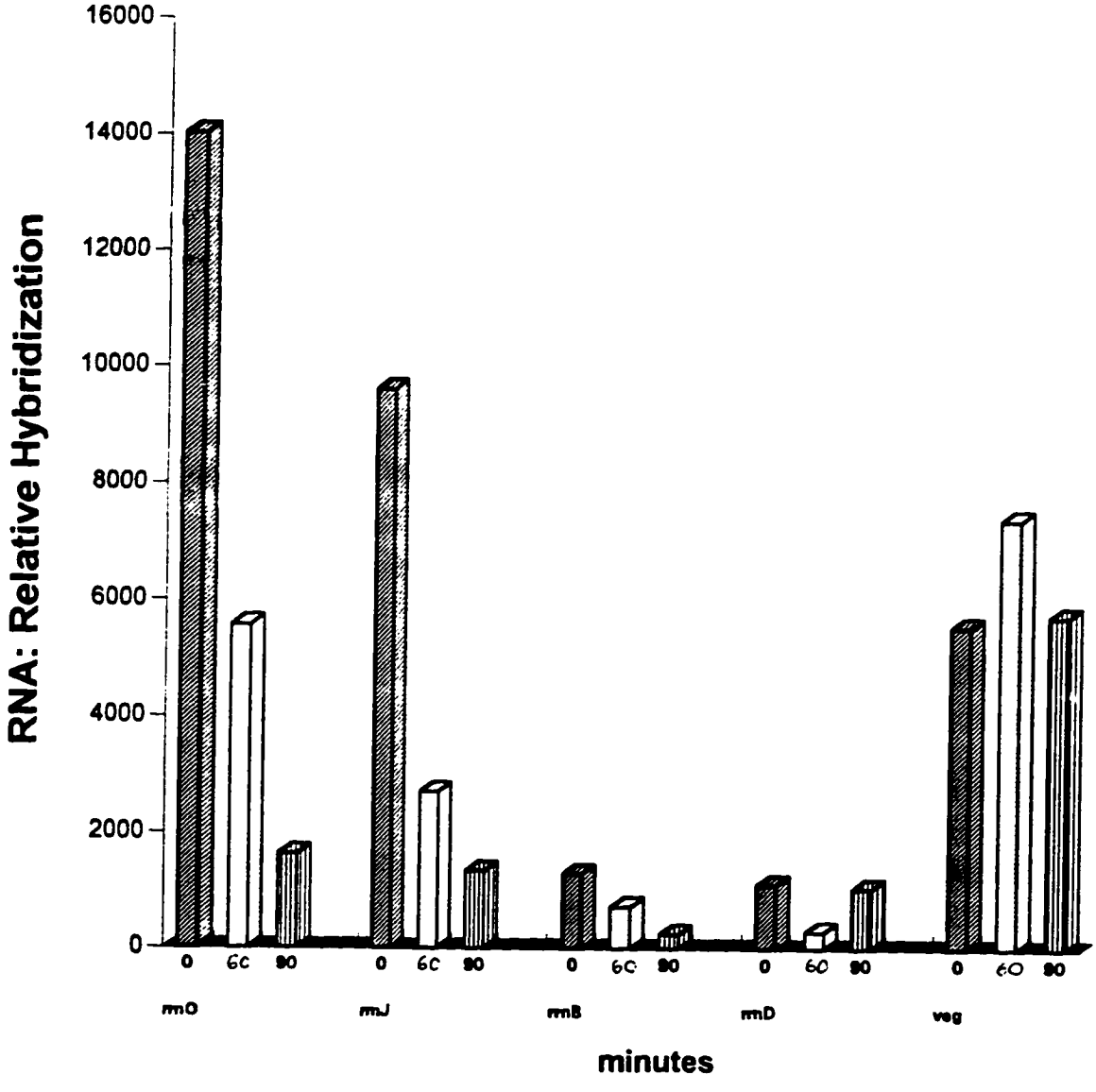


Fig. 17. The effect of serine hydroxamate (SHX) on the expression of the *rrnO* promoters

The effect of serine hydroxamate (SHX) on the expression of the *rrnO*-P1P2, *rrnO*-P1, and *rrnO*-P2, *lacZ* fusions (pPW4, pLR501, and pLR512) in *B. subtilis relA* wild-type and mutant strains (IS58, IS56). The relative activities of the promoters were determined by β- galactosidase Miller units and RNA dot blot analysis. Bacteria were grown in MM-Glu medium and SHX was added to the cultures when the Klett reached 80 to 85. Samples were taken at 0, 30, and 60 minutes after addition of 2 mg/ml SHX to the cultures. The levels of *lacZ* mRNA in total RNA were determine by hybridization to probe specific for *lacZ* mRNA. The probe was labeled with the random primer extension kit, using α-^{32}P-dCTP (Samarrai et al., 1996).

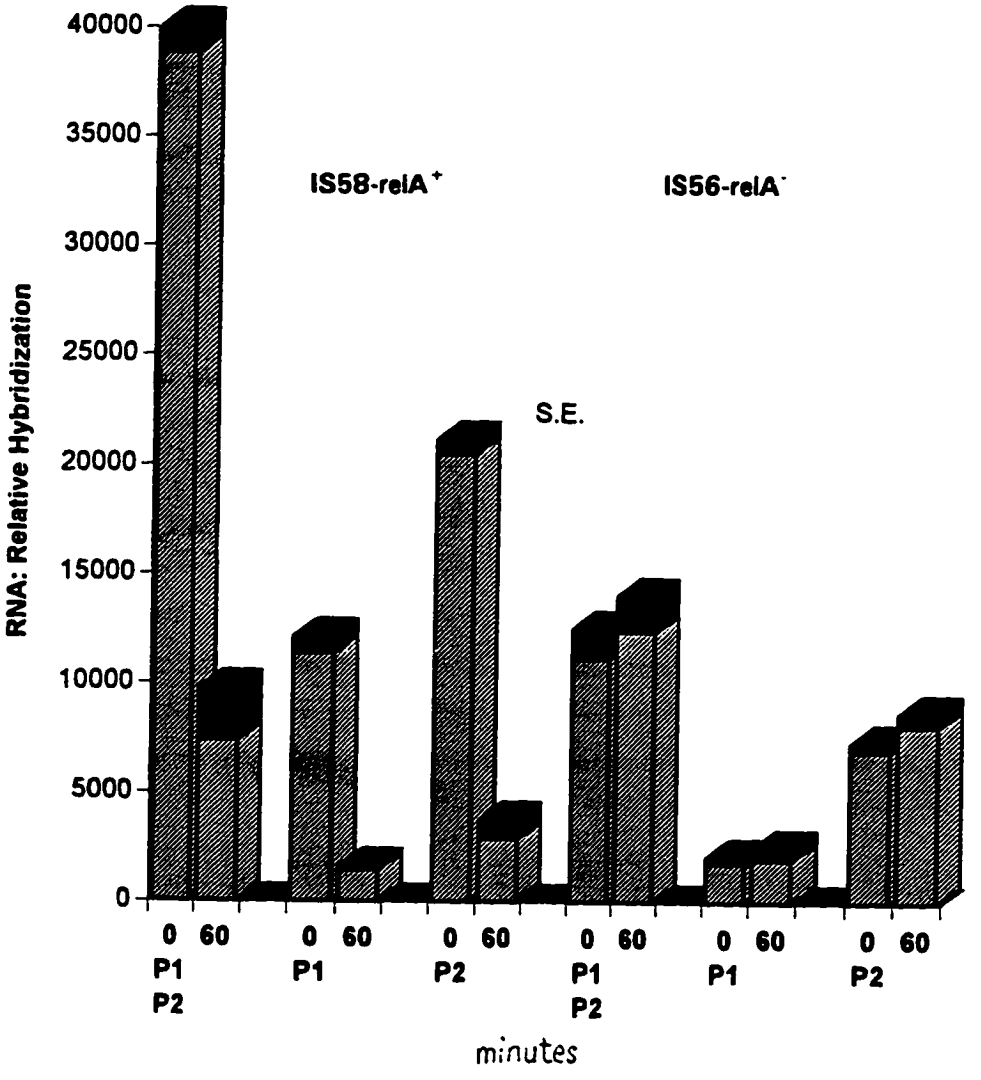


Fig. 18. The effect of serine hydroxamate (SHX) on the expression of the *rrnJ* promoters

The effect of serine hydroxamate (SHX) on the expression of *rrnJ*-P1P2, *rrnJ*-P1, and *rrnJ*-P2, *lacZ* fusions (pAWR108, pLR210, pLR105) in *B. subtilis relA* wild-type and mutant strains (IS58, IS56). The relative activities of the promoters were determined by β-galactosidase Miller units and RNA dot blot analysis. Bacteria were grown in MM-Glu medium and SHX was added to the cultures when the Klett reached 80 to 85. Samples were taken at 0, 30, and 60 minutes after addition of 2 mg/ml SHX to the cultures. The levels of *lacZ* mRNA in total RNA were determine by hybridization to probe specific for *lacZ* mRNA. The probe was labeled with the random primer extension kit, using α-^{32}P-dCTP (Samarrai et al., 1996).

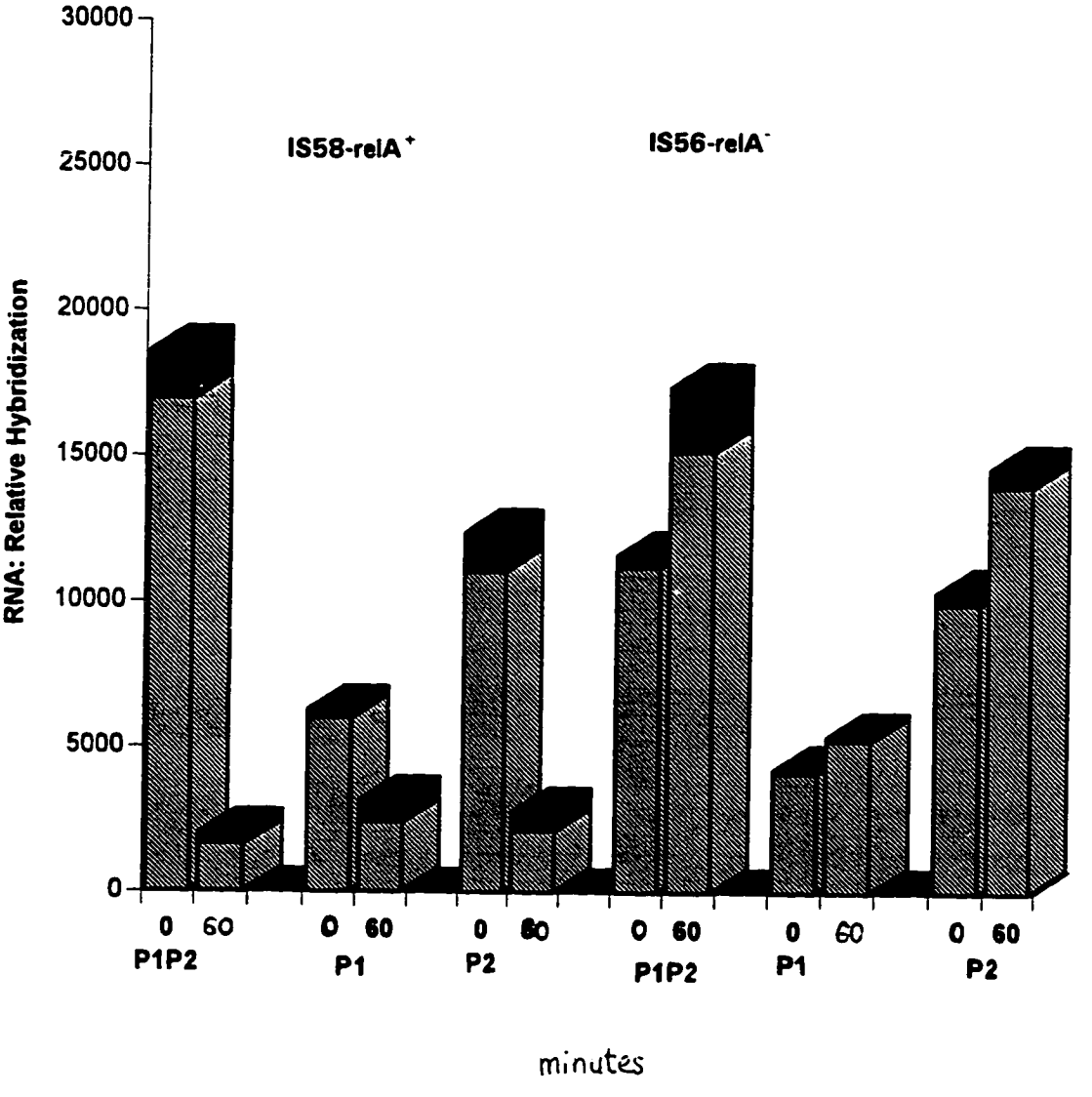


Fig. 19. The effect of serine hydroxamate (SHX) on the expression of *B. subtilis rrn* promoters

The effect of serine hydroxamate (SHX) on the expression of the *rrnO*-P1P2, *rrnJ*-P1P2, *rrnB*-P1P2, *rrnD*-P1P2, and *veg* (control), *lacZ* fusions (pPW4, pAWR108, pAWR123, and pAWR116, and pPW810) in *B. subtilis relA* wild-type and mutant strains (IS58, IS56). The relative activities of the promoters were determined by β-galactosidase Miller units and RNA dot blot analysis. Bacteria were grown in MM-Glu medium and SHX was added to the cultures when the Klett reached 80 to 85. Samples were taken at 0, 30, and 60 minutes after addition of 2 mg/ml SHX to the cultures. The levels of *lacZ* mRNA in total RNA were determine by hybridization to probe specific for *lacZ* mRNA. The probe was labeled with the random primer extension kit, using α-^{32}P-dCTP (Samarrai et al., 1996).

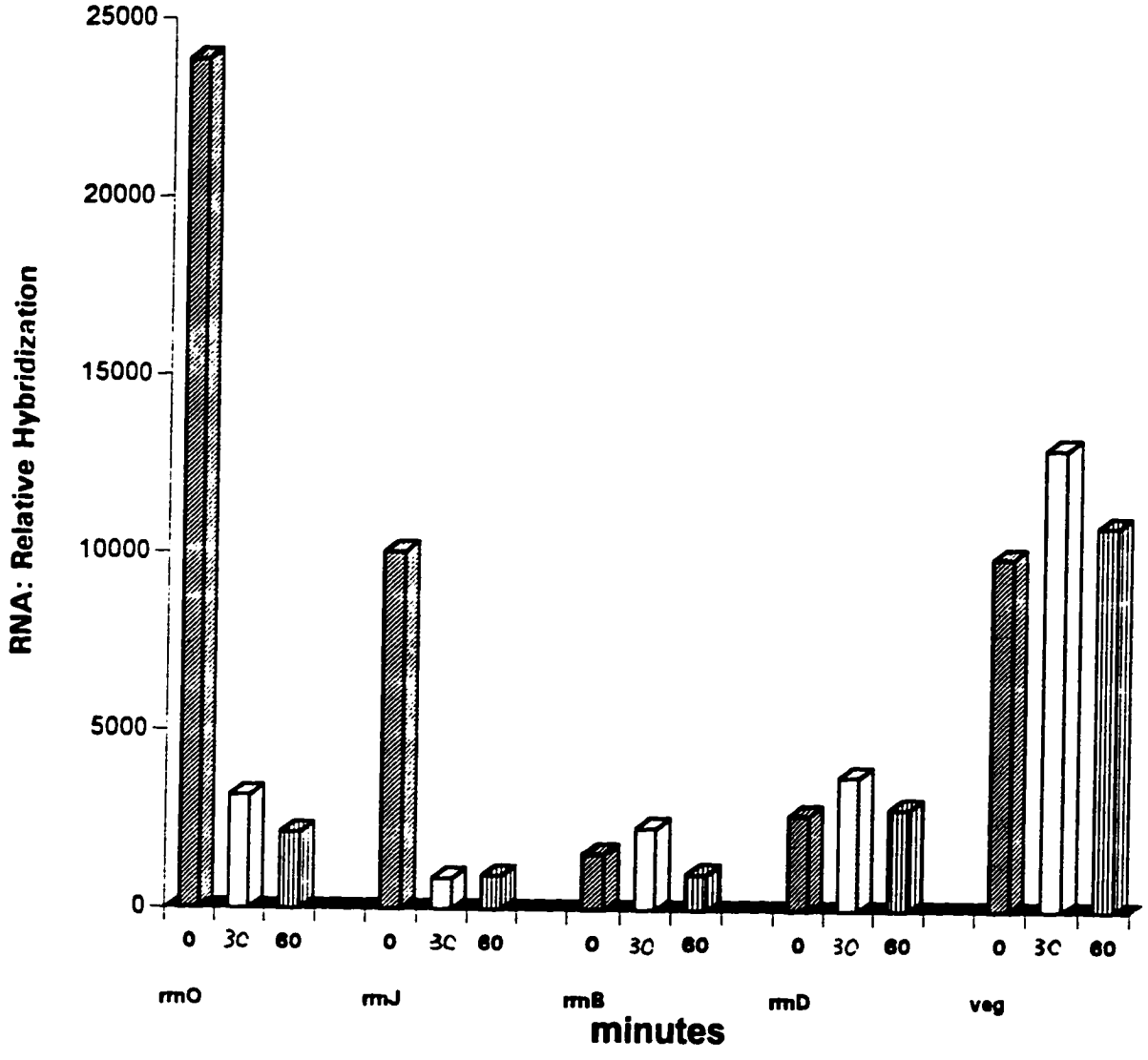


Fig. 20. Fis-binding consensus sequences found in the *B. subtilis rrnO* UAS region

Fis consensus sequence was taken from Hubner and Arber (1989) and *rrnO* sequence was from Ogasawara et al. (1983a). Thirteen Fis sequences were found in *rrnO*, 7 in *rrnD*, and 5 in *rrnJ* as well as *rrnB*. The core consensus sequences of P1 and P2 promoters and the transcription start site are marked with boldfaced letters and underlined -35, -10, and +1. The sequences matched with Fis consensus sequence are underlined and numbered with # marks beginning from the 16S rRNA structural gene towards upstream. The beginning of the 16S rRNA gene is marked with double-underlines. Only the sequences which are covered by the PCR fragments *rrnO*-UAS, *rrnO*-P1, and *rrnO*-P2, are given. The regions complementary to relevant primers are labeled and marked with dotted-line arrows and the numbers of the primers used.

R15-80

GGAATTCA ATGCGGAGCT TTACCTCTTC TTCACTACAA

AGCATGGGGT TTCAAAACGA ACATCGCTAT CTCAATTCGC TAATATCCGC AACAATGGTC TAATTGCTCT

GAGTCTTCGT GAAGATGATG AACTGATGGG TGTACGTCTG ACTGACGGCA CAAAACAAAT CATCATTGGA

#10

ACGAAAAACG GTTTACTGAT TCGTTTCCCT GAAACAGATG TCCGAGAGAT GGGAAGAACT CCGGCGGGCG

#9

TAAAAGGCAT CACCCTGACG GATGACGACG TTGTTGTCGG CATGGAGATT TTAGAGGAAG AATCACACGT

#8

ACGAACTCCT CCTTATCGTA ACTGAAAAAG GGTACGGAAA ACGAACTCCT GCTGAAGAGT ACAGAACCCA

#7

AAGCCGGGGC GGAAAAGGAC TCAAAACAGC GAAAATCACC GAGAACAACG GCCAACTAGT AGCAGTGAAA

#6

GCTACTAAAG GTGAAGAGGA TCTAATGATT ATTACAGCTA GCGGCGTACT CATCAGAATG GACATCAATG

R15-81 R15-79

ATATCTCCAT CACCGGACGT GTCACTCAAG GTGTGCGTCT CATCAGAATG GCAGAAGAAG AGCATGTTGC

TACAGTAGCT TTAGTTGAGA AAAACGAAGA AGATGAGAAT GAAGAAGAAC AAGAAGAAGT GTGAAAAAAA

(-35 P1 -10)

GCGCAGCTGA AATAGCTGCG CTTTTTTGTG TCATAACCCT TTACAGTCAT AAAAATTATG GTATAATCAT

#4

+1 R15-78 R15-77 (-35

TTCTGTTGTC TTTTTAAAGA CACAAGCATG ACCATTATGA CTAGTAAAAA CTTTTTCAAA AAAGTATTGA

#3

P2 -10) +1

CCTAGTTAAC TAAAAATGTT ACTATTAAGT AGTCGCTTTG AGAGAAGCAC ACAAGTTCTT TGAAAACTAA

#2

ACAAGACAAA ACGTACCTGT TAATTCATTT TTATAAATCG CACAGCGATG TGCGTAGTCA GTCAAACTAG

#1

GGCCTGCACG ACGCAGGTCA CACAGGTGTC GCCGCAGGAT GCGGTGAACT TAACCTGTGA TCCATTTATC

R15-71

GGAGAGTTTG ATCCTGGCTC AGGACGACGC TGGCGGCGTG CCTAATACAT GCAAGTCGAG CGGACAGATG

16S rRNA

Fig. 21. Analysis of the *rrnO*-UAS-binding properties in *B. subtilis* crude protein extracts and a comparison to the *E. coli* Fis protein

(a) Restriction digested and ^{32}P-labeled *rrnO*-UAS (549 bp) was incubated with *E. coli* Fis protein or *B. subtilis* crude protein extracts prior to separation on an 8% acrylamide gel. 0.3 ng labeled DNA fragments was incubated with 2-20 ng of *E. coli* Fis or 0.1-0.5 µg *B. subtilis* protein preparations at 37^{0}C for 10 minutes. Lanes 1-7: protein extracts of *B. subtilis* growing stages #1, 2, 3, 4, 5, 6, and 8, as labeled in (b); Lane 8: no protein; Lanes 9 and 10: *E. coli* Fis protein 20 and 2 ng, respectively.

(b) Isolation of crude protein extracts from *B. subtilis* strain IS58 at different times during growth. Overnight culture (in MM-Glu) of *B. subtilis* IS58 cells were harvested and inoculated into prewarmed VY to Klett 30. The culture was grown at 37^{0}C shaker and aliquots of 600 ml (at low Klett in the early phase of growth) to 100 ml (at high Klett) were drawn at different times during growth as indicated at #1 to #8. #1: 600 ml at Klett reading 32; #2: 600 ml at Klett reading 34; #3: 500 ml at Klett reading 68; #4: 400 ml at Klett reading 105; #5: 300 ml at Klett reading 173; #6: 200 ml at Klett reading 231; #7: 100 ml at Klett reading 268; #8: 100 ml at Klett reading 272. Crude protein extracts were prepared as described in the Materials and Methods.

(a)

(b)

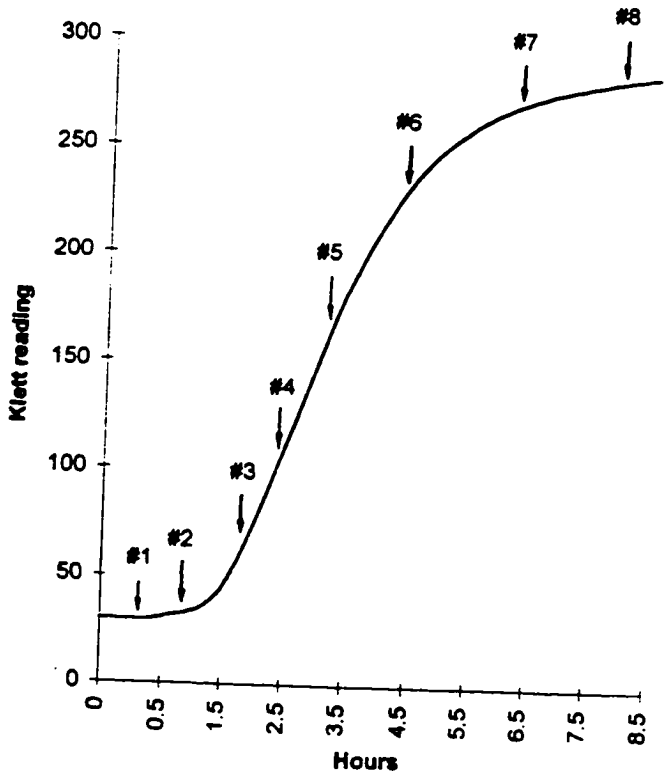


Fig. 22. Binding of the *E. coli* Fis and *B. subtilis* proteins (HB-fraction preparations) to *rrnO*-UAS

B. subtilis protein samples used were the #3 and #6 preparations showed in Fig. 21. Lane 1: labeled 1kb ladder; Lane 2: no protein; Lanes 3 and 4: 0.5 and 5 ng *E. coli* Fis; Lanes 5-7: 0.5, 1.5, and 2.5 µg #6; Lanes 8-10: 0.1, 0.3, and 0.5 µg #3. DNA probe used was the *rrnO*-UAS (with an equal molar vector plasmid pGEM-Cat which formed a band at the top). Binding condition was the same as mentioned in Fig. 21. Free DNA bands are labeled as F; complexes are labeled as C1 and C2.

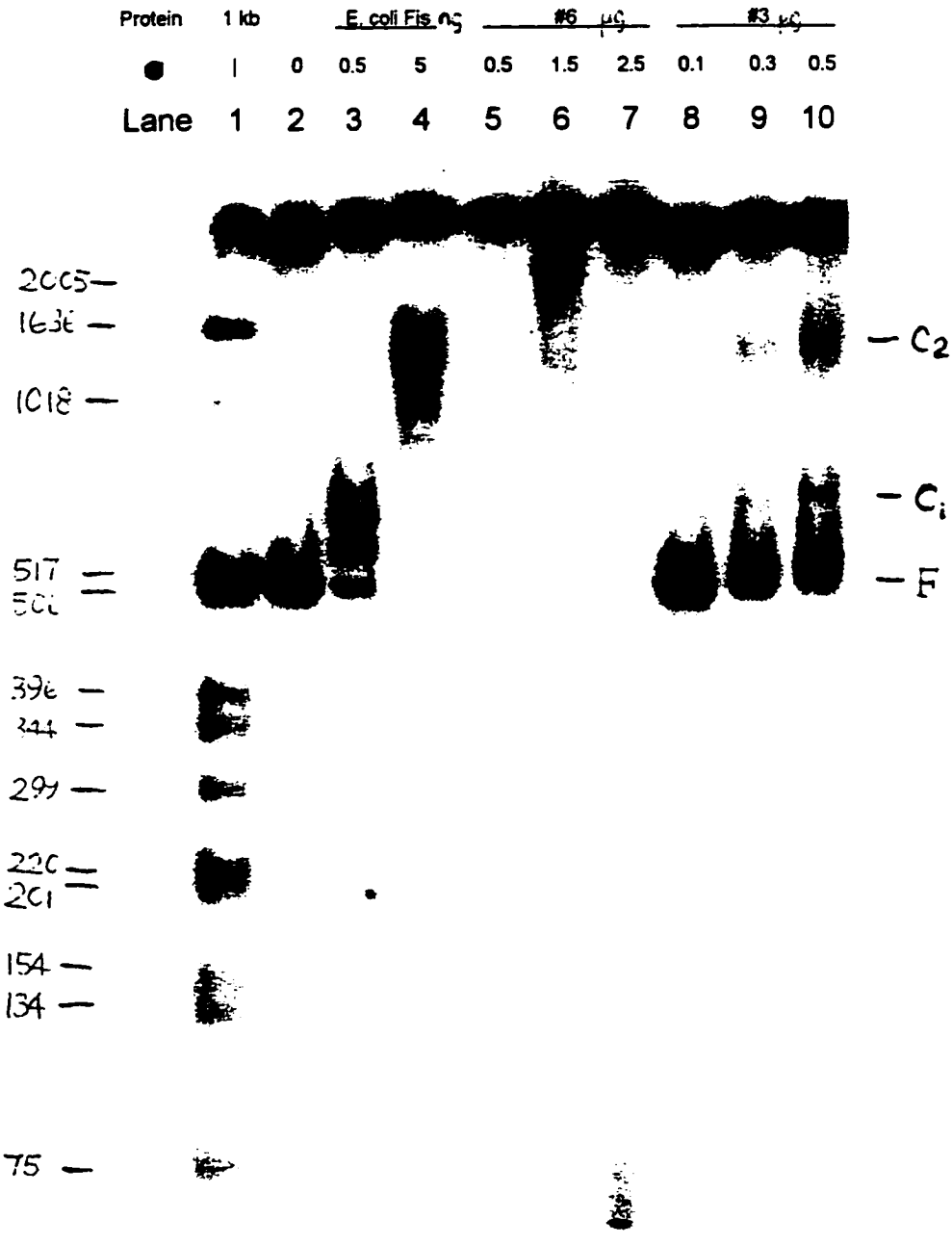


Fig. 23. Binding of *E. coli* Fis to *rrnJ*-P2 as a function of *E. coli* Fis concentration

Restriction digested and ^{32}P-labeled *rrnJ*-P2 (269 bp, with an equal molar vector plasmid pGEM-Cat which formed a band at the top) was incubated with various amounts of *E. coli* Fis protein as indicated prior to electrophoresis on an 8% acrylamide gel. 0.6 ng labeled *rrnJ*-P2 was incubated with 0.1-16 ng of *E. coli* Fis at 37^{o}C for 10 minutes. Lanes 1-15: 0.3, 0.2, 0.1, 0, 1.6, 1.2, 0.8, 0.4, 0, 2.0, 4.0, 8.0, 12.0, 16.0, and 0 ng of *E. coli* Fis protein, respectively.

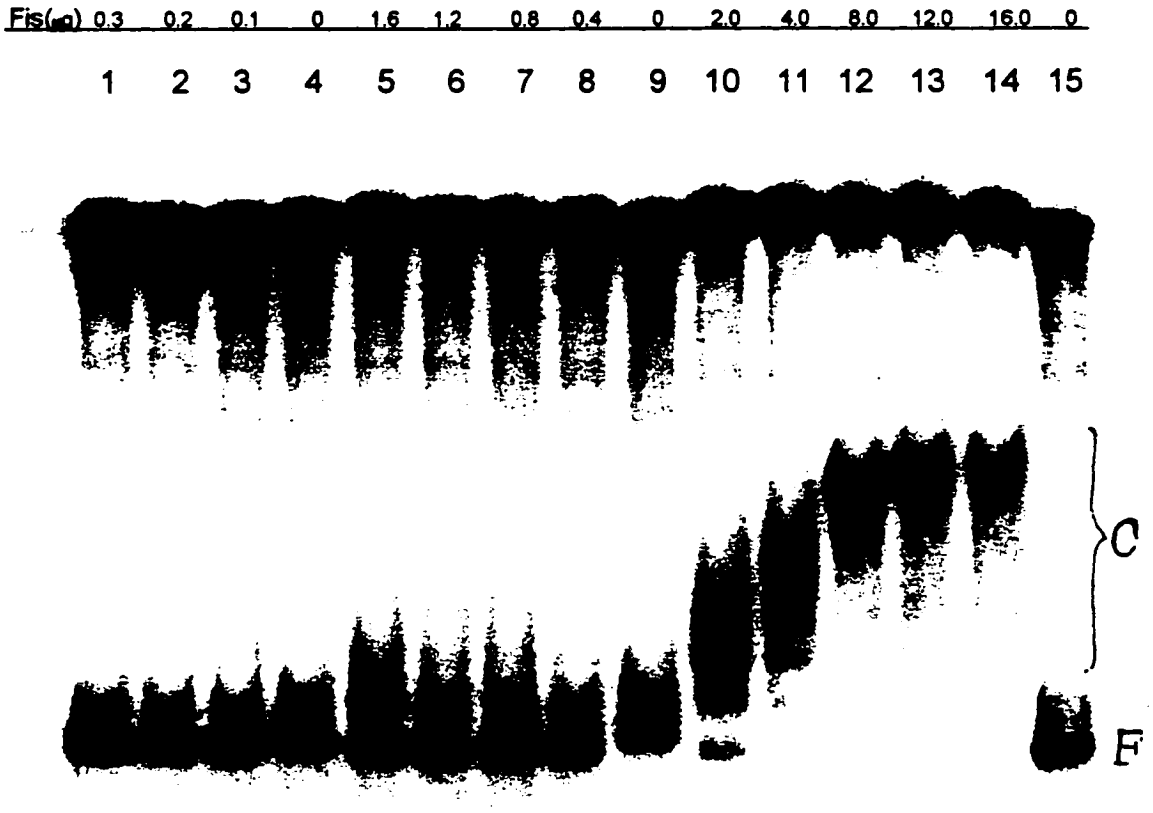


Fig. 24. Comparison of the binding of *E. coli* Fis to *rrnO*-UAS, *rrnJ*-P1, and *rrnJ*-P1P2

0.3 ng of restriction digested and ^{32}P-labeled *rrnO*-UAS (549 bp), *rrnJ*-P1 (194 bp), and *rrnJ*-P1P2 (441 bp) (all samples contained an equal molar vector plasmid pGEM-Cat which formed a band at the top) were incubated with various amounts of *E. coli* Fis protein as indicated prior to electrophoresis on an 8% acrylamide gel. Lanes 1-3: 0.3 ng *rrnO*-UAS DNA; Lanes 4-8: 0.2 ng *rrnJ*-P1; Lanes 9-15: 0.2 ng *rrnJ*-P1P2. For the amount of *E. coli* Fis protein: Lane 1-3: 0, 2 and 6 ng, respectively; Lane 4-8: 0, 4, 8, 12, and 16 ng, respectively; Lane 9-15: 0, 2, 4, 6, 8, 12, and 16 ng, respectively.

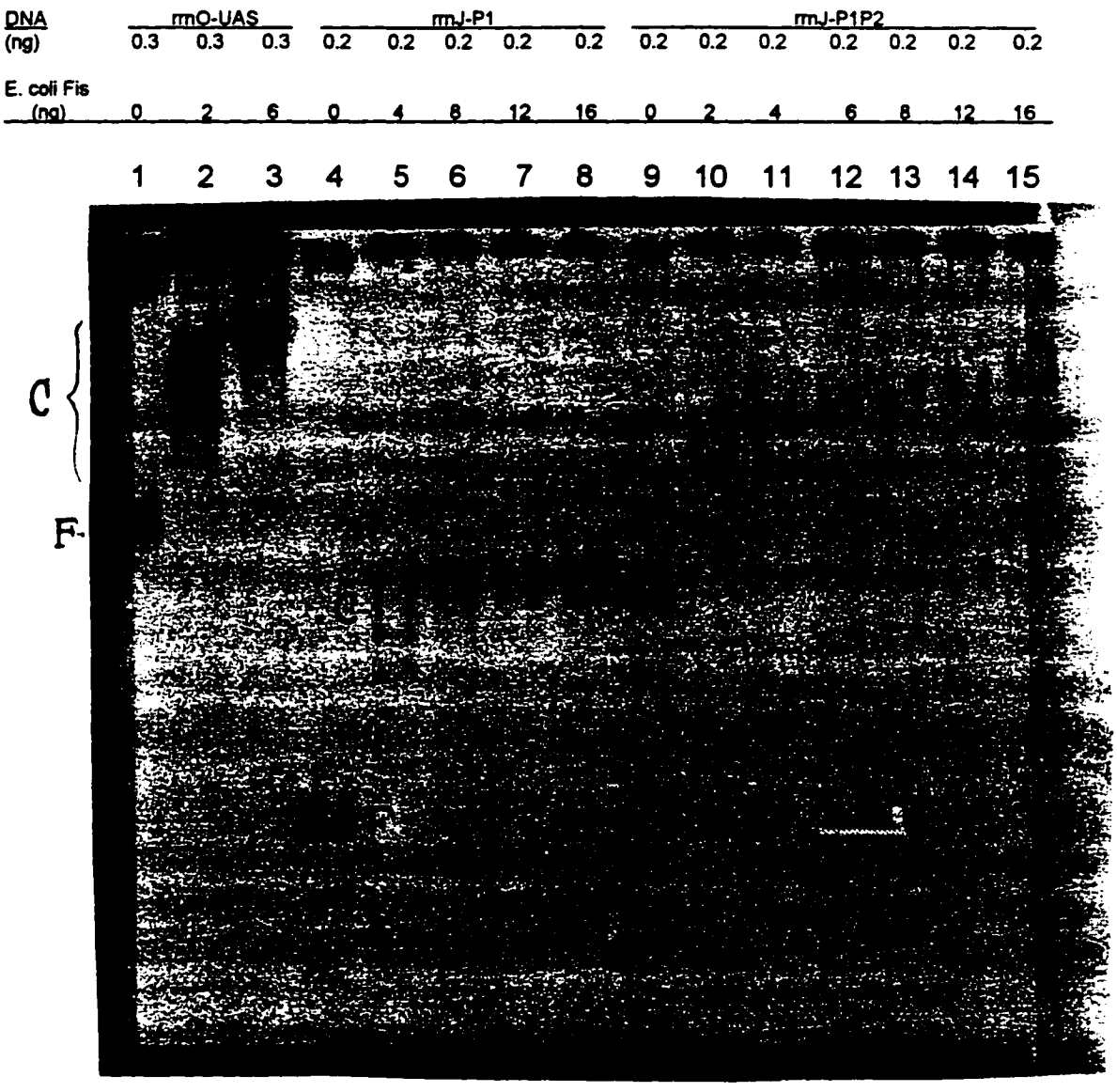


Fig. 25. Binding of Fis protein to the structural gene of *B. subtilis* 23S rRNA

Restriction digested (HindIII and BamHI) and ^{32}P-labeled *B. subtilis* 23S rRNA gene fragment (315 bp; Jarvis et al., 1990) was incubated with *E. coli* Fis protein prior to separation on an 8% acrylamide gel. 0.4 ng labeled DNA fragments were incubated with 20-100 ng of *E. coli* Fis protein at 37^{0}C for 10 minutes. Lane 1-6: DNA with *E. coli* Fis protein 0, 20, 40, 60, 80, and 100 ng, respectively. F: free 23S rRNA gene fragment; C: complexes formed by Fis protein and the rRNA gene fragment.

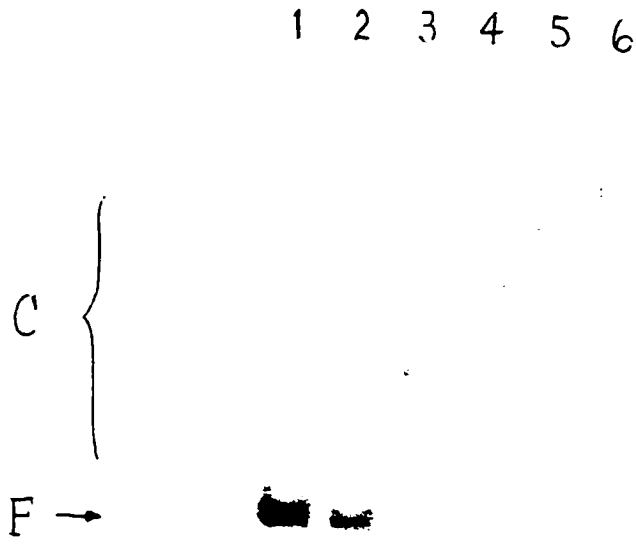
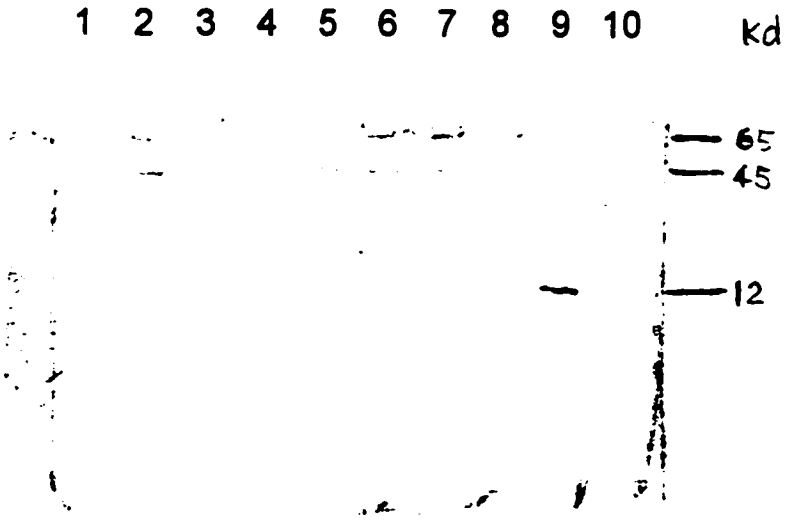


Fig. 26. Western blots: Analysis of homology between *E. coli* Fis protein and *B. subtilis* protein

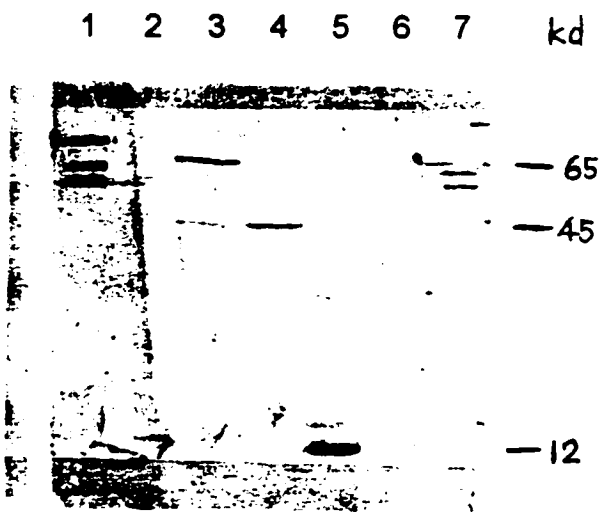
0.2 to 5 µg of *B. subtilis* HB-protein preparations or crude protein extracts (depending on the concentration of the protein samples) or 0.1 µg *E. coli* Fis protein were separated on an 8% SDS polyacrylamide gel and transferred onto nitrocellulose membranes using a MiniBlot-SDE (Millipore). The western blots (immunoblots) were first incubated in 5% milk at room temperature on a rocking bed for 2 hrs. The membranes were washed in TBE buffer for 1 hour and then transferred to a 1:1000 dilution of rabbit anti-Fis serum (Ball et al., 1992) for another 2 hr-incubation. After 4 periods of 15 minutes washing the membranes were incubated with horseradish peroxidase-labeled goat anti-rabbit (Materials and Methods). The films were developed using an ECL western blotting kit following the procedure of the kit provider.

a) Western blot for crude protein extracts. Lanes 1: #3 crude protein extraction; Lane 2: crude protein extraction isolated from early log phase in another experiment; Lane 3-8: #1-#6 protein preparations (Fig. 21); Lane 9: 100 ng *E. coli* Fis; and Lane 10: 2 ng *E. coli* Fis. b) Western blot for HB protein preparations of #3 protein. Lane 1: *E. coli* total protein (stationary phase); Lane 2: *E. coli* total protein (early log phase); Lane 3: crude protein extract #3; Lane 4: HB protein preparation of #3 protein extract; Lane 5 and 6: 100 ng and 5 ng Fis protein, respectively. c) Coomassie blue stained gel. Lane 1: Fis protein; Lanes 2-9: crude protein preparations #1-#8; Lane 10: HB fraction of crude protein preparation #3.

(a)



(b)



(C)

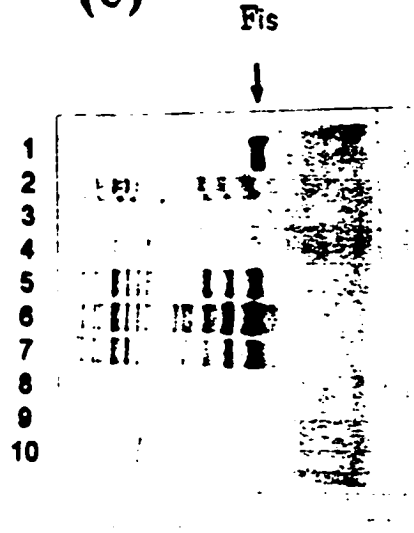
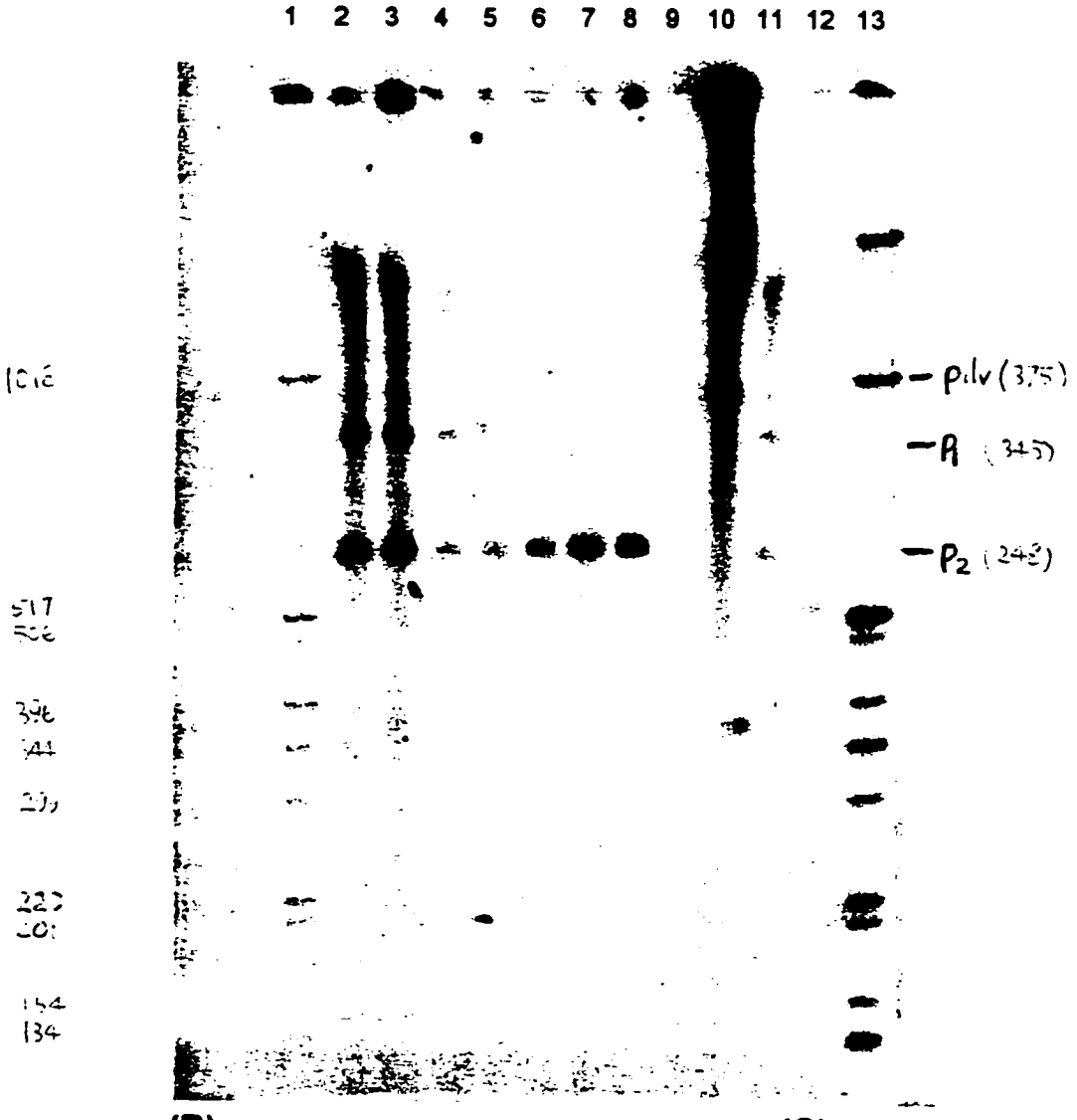


Fig. 27. Activities of P1 and P2 promoters on the *rrnO*-P1P2 template as a function of NaCl concentrations

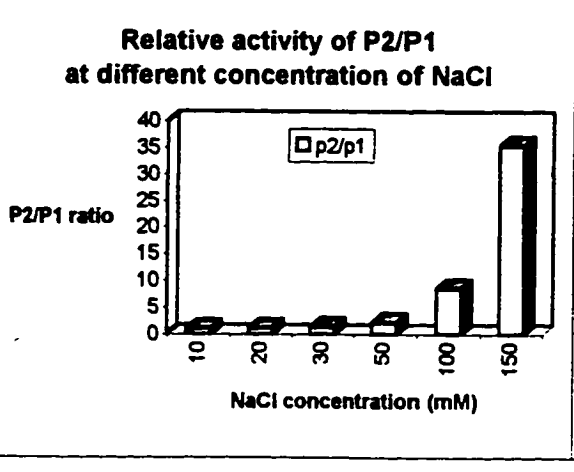
Reactions were initiated by addition of RNAP, incubated (37^0 C) for 16 minutes in reaction buffer containing indicated amount of NaCl, and terminated with stop solution (2.5 M NH_4OAc, 20 mM EDTA, and 100 µg/ml carrier plasmid DNA). A) autoradiogram. Numbers in parenthesis are the numbers of nucleotides of transcripts; P1, 345; P2, 248; and pilV, 375 nucleotides. Lane 1, 1 kb DNA ladder*; Lanes 2-8, NaCl concentration of 10, 20, 30, 50, 100, 150, and 200 mM, respectively; Lane 9, 10 ng pPW4 (PCR template for *rrnO* promoters) used as a control, 50 mM NaCl; Lane 10, pilV promoter, 50 mM NaCl; Lane 11, repeat of lane 5; Lane 12, labeled *rrnO*-UAS; Lane 13, repeat of lane 1. B) P2/P1 ratio at different concentrations of NaCl. C) comparison of the P1 and P2 activities at low (10 and 20 mM) and high (150 and 200 mM) NaCl.

* DNA fragments in 1 kb DNA ladder migrate differently from the rRNA transcripts of similar sizes; so its bands served only as landmark references for the RNA transcripts.

(a)



(B)



(C)

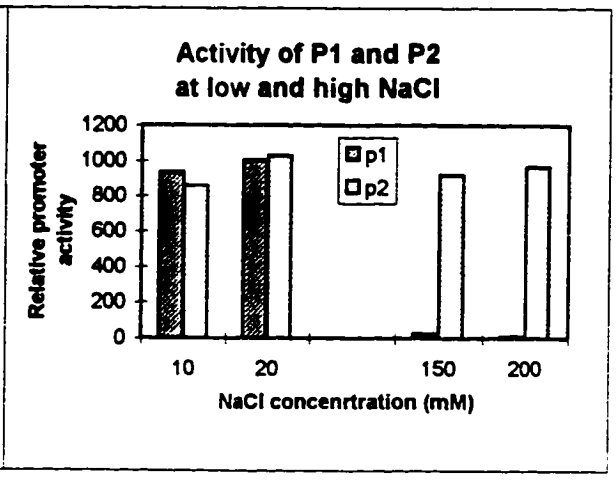
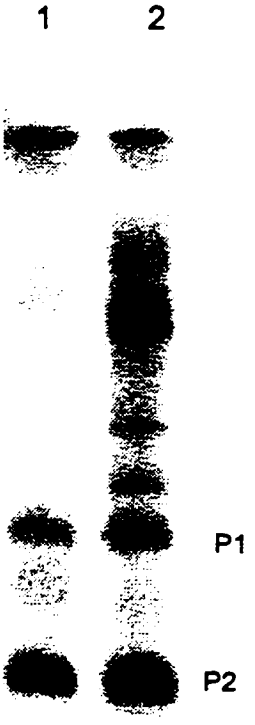


Fig. 28. *In vitro* transcription at 50 mM of *rrnO*-P1P2 with or without UAS

0.1 pmoles of DNA templates (*rrnO*-UAS-P1P2 or *rrnO*-P1P2, Table 3) and 0.5 pmoles *B. subtilis* RNAP were used in the reactions (Materials and Methods). Reactions were initiated by addition of RNAP, incubated (37^0 C) for indicated time period, and terminated with stop solution (2.5 M NH_4OAc, 20 mM EDTA, and 100 µg/ml carrier calf thymus DNA or plasmid DNA). Transcripts were separated by electrophoresis on denaturing 6% polyacrylamide-7M urea gels and visualized by autoradiography. Quantitation was done by densitometry using Molecular Dynamics phosphor imaging system. The molar ratios of transcripts were calculated by correcting for the number of UMP residues incorporated into each run-off transcripts. (A) A sample of run-off assay. Lane 1: *rrnO*-UAS-P1P2; lane 2: *rrnO*-P1P2. (B) Data were obtained and calculated from seven independent experiments.

(A)



(B)

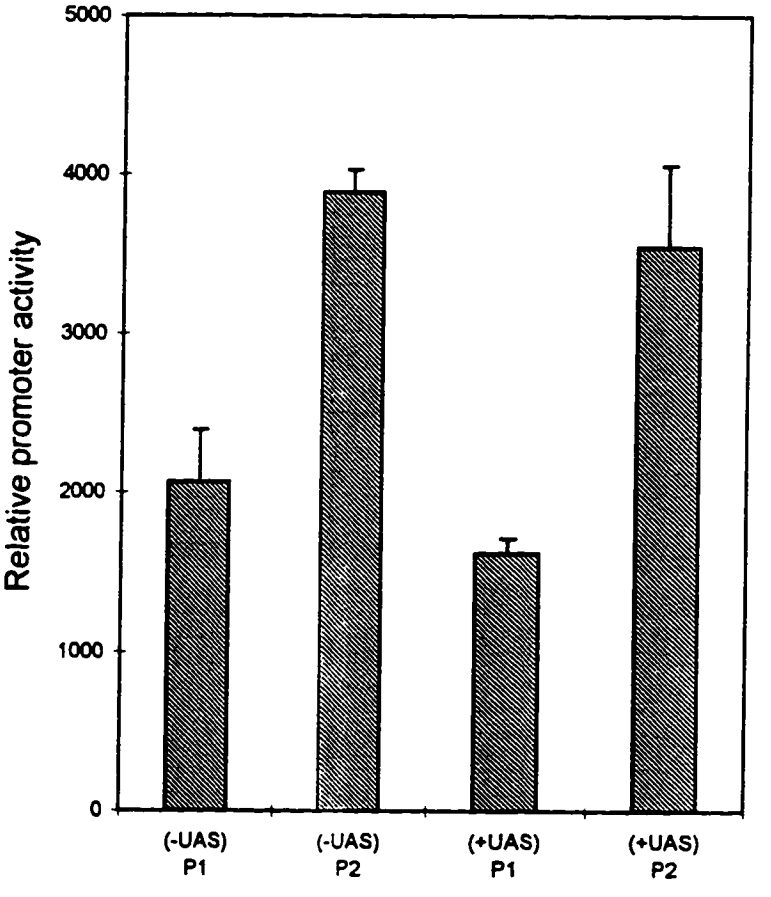


Fig. 29. *In vitro* transcription at 150 mM of *rrnO*-P1P2 with or without UAS

0.1 pmoles of DNA templates (*rrnO*-UAS-P1P2 or *rrnO*-P1P2, Table 3) and 0.5 pmoles *B. subtilis* RNAP were used in the reactions. Reactions were initiated by addition of RNAP, incubated (37^0 C) for indicated time period, and terminated with stop solution (2.5 M NH_4OAc, 20 mM EDTA, and 100 µg/ml carrier plasmid DNA). Transcripts were separated by electrophoresis on denaturing 6% polyacrylamide-7M urea gels and visualized by autoradiography. Quantitation was done by densitometry using Molecular Dynamics phosphor imaging system. The molar ratios of transcripts were calculated by correcting for the number of UMP residues incorporated into each run-off transcripts. (A) A sample of run-off assay. Lane 1: *rrnO*-UAS-P1P2; lane 2: *rrnO*-P1P2. (B) Data were obtained and calculated from seven independent experiments.

(A)

(B)

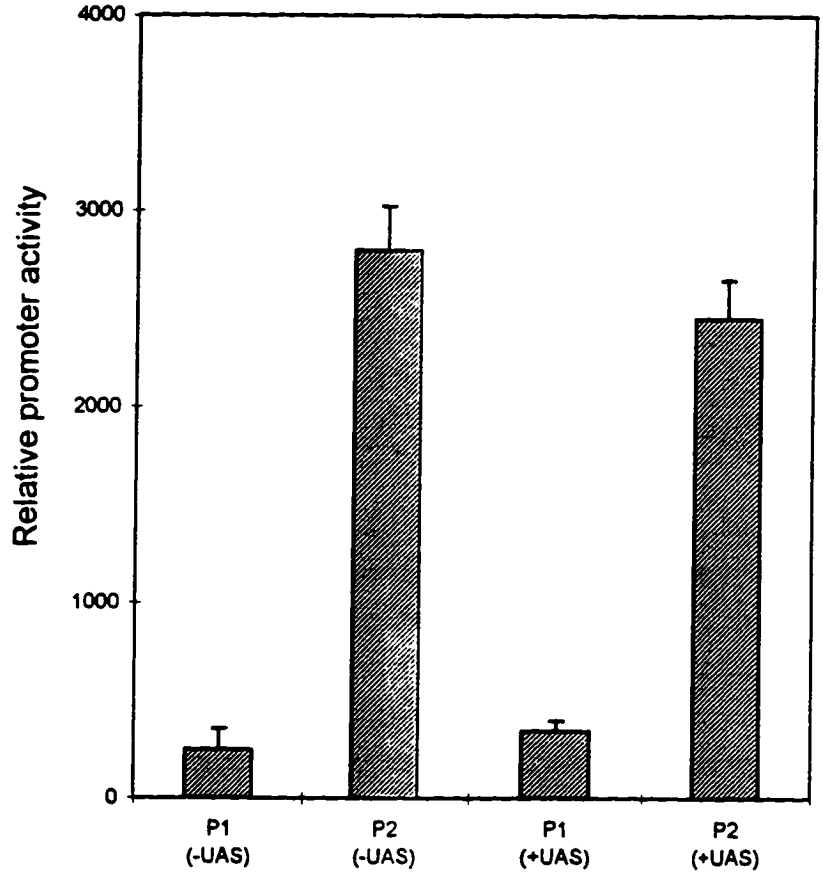


Fig. 30. *In vitro* transcription of *rrnO*-P1 and *rrnO*-P2 at 50 mM and 150 mM NaCl

0.1 pmoles of DNA templates and 0.5 pmoles *B. subtilis* RNAP were used in the reactions. Reactions were initiated by addition of RNAP, incubated (37°C) for 16 minutes, and terminated with stop solution (2.5 M NH_4OAc, 20 mM EDTA, and 100 µg/ml carrier plasmid DNA). Transcripts were separated by electrophoresis on denaturing 6% polyacrylamide-7M urea gels and visualized by autoradiography. Quantitation was done by densitometry using Molecular Dynamics phosphor imaging system. The molar ratios of transcripts were calculated by correcting for the number of UMP residues incorporated into each run-off transcripts. (A) A sample of run-off assay. Because the *rrnO*-P1 transcript is only 49 nucleotides long, the samples in lanes 3 and 4 were loaded on gel much later than those in lanes 1 and 2. The intensities of the *rrnO*-P1 transcripts were obtained after subtracting the backgrounds of the areas lower than but close to the positions of the P1 transcripts. Lane 1-4: *rrnO*-P2, 50 mM NaCl; *rrnO*-P2, 150 mM NaCl; *rrnO*-P1, 50 mM NaCl; *rrnO*-P1, 150 mM NaCl. (B) Data were obtained and calculated from six independent experiments.

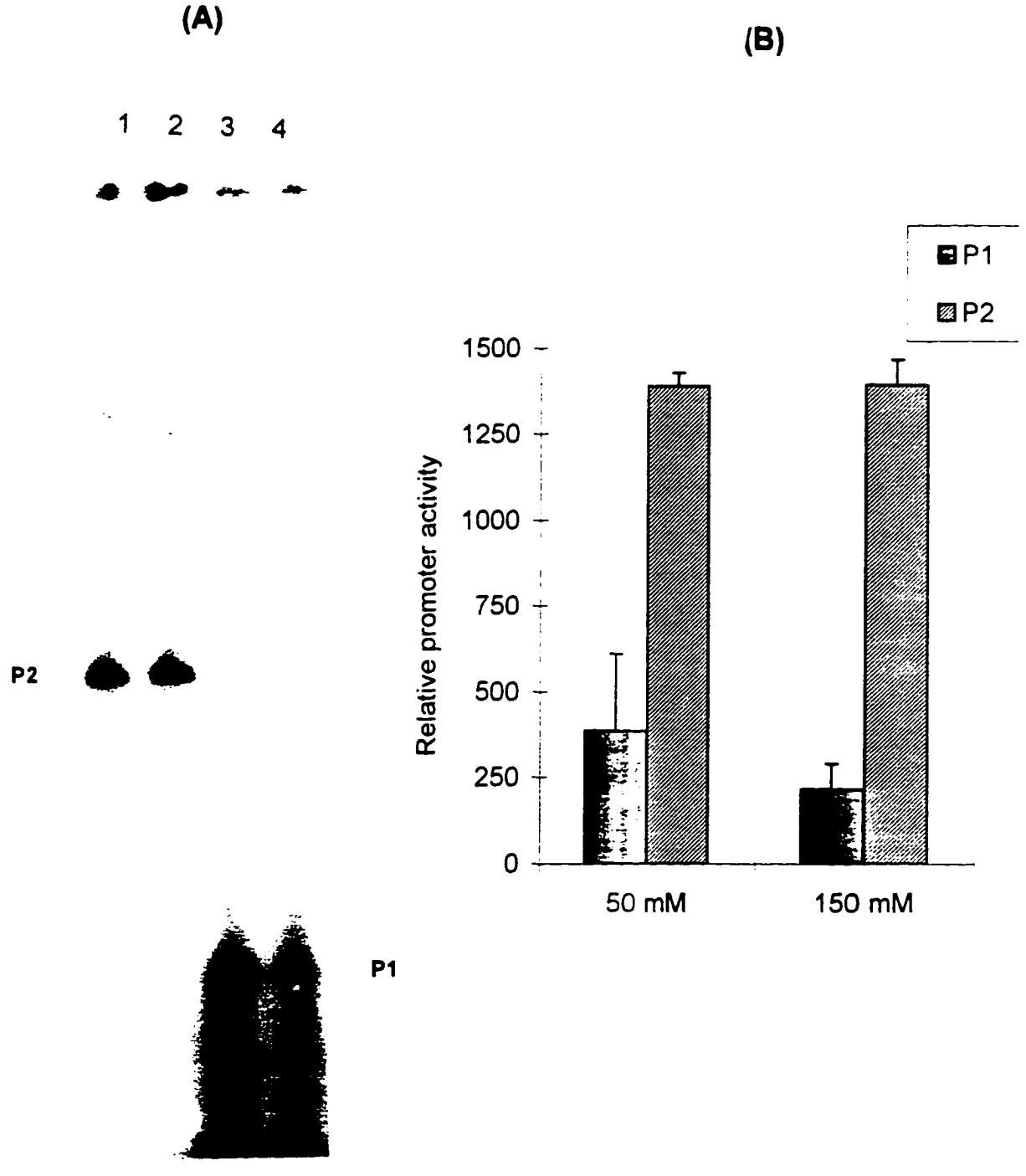


Fig. 31. *In vitro* transcription of *rrnJ*-P1P2 and *rrnJ*-P1(H)P2 promoters

0.2 pmoles of DNA templates and 0.5 pmoles *B. subtilis* RNAP were used in the reactions. Transcription reactions were carried out in transcription buffer containing 10 mM NaCl. Reactions were initiated by addition of RNAP, incubated (37° C) for indicated time period, and terminated with stop solution (2.5 M NH_4OAc, 20 mM EDTA, and 100 µg/ml carrier plasmid DNA). After extraction with phenol/$CHCl_3$, the nucleic acids in samples were ethanol-precipitated before electrophoresis on denaturing 6% polyacrylamide-7M urea gels. Individual transcripts were visualized by autoradiography. Quantitation was done by densitometry using a Molecular Dynamics phosphor imaging system. The molar ratios of transcripts were calculated by correcting for the number of UMP residues incorporated into each run-off transcripts. Lanes 1 and 2: *rrnJ*-(-UAS)-P1P2 and *rrnJ*-(-UAS)-P1(H)P2, 8 minutes reaction; lanes 3 and 4: *rrnJ*-(-UAS)-P1P2 and *rrnJ*-(-UAS)-P1(H)P2, 16 minutes reaction

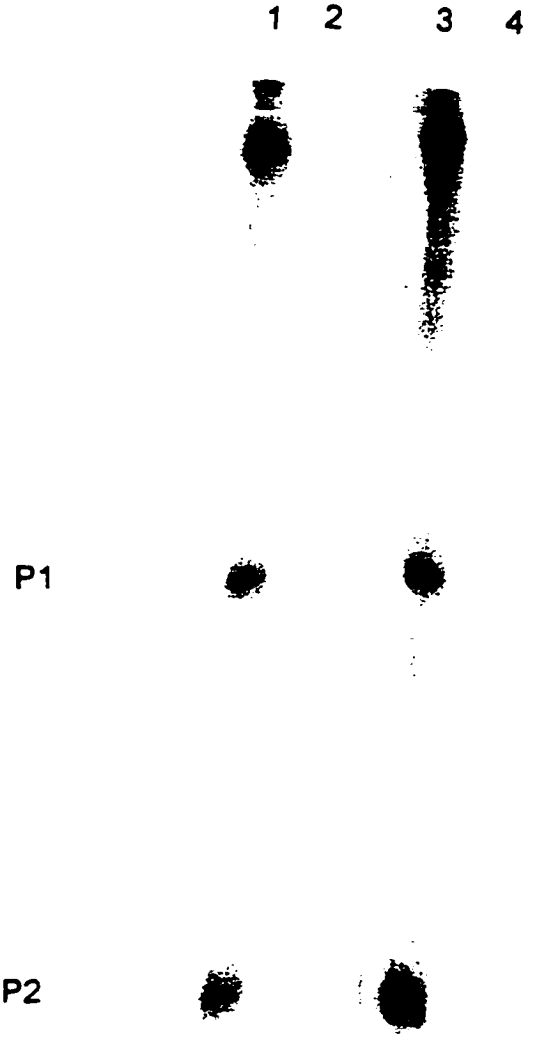
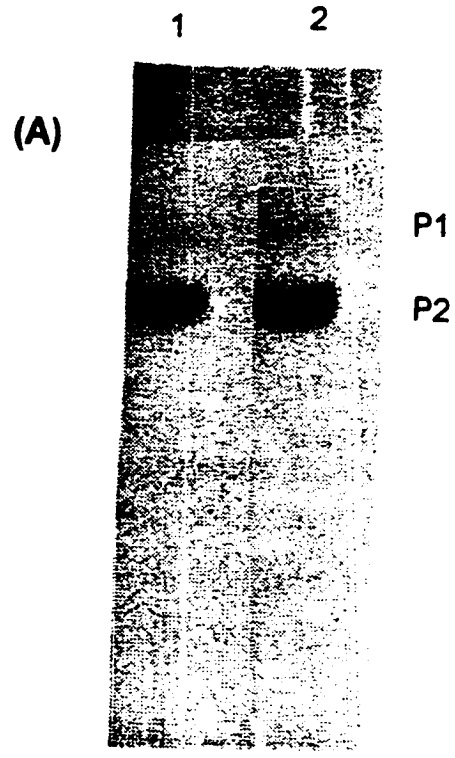
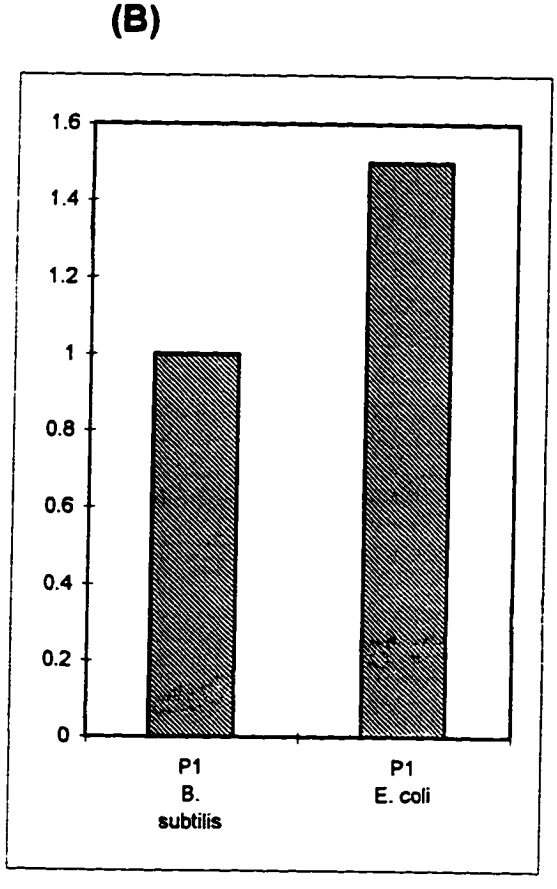
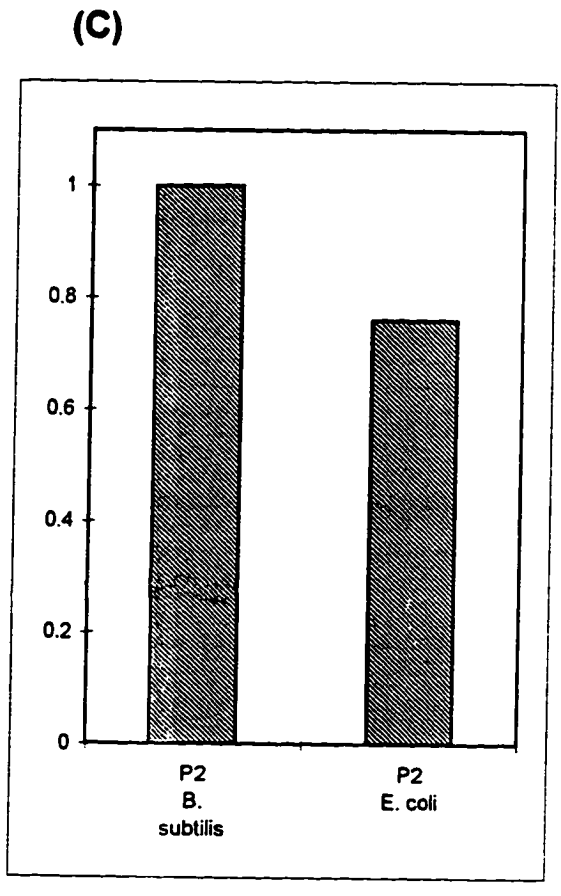


Fig. 32. Comparison of *B. subtilis* RNAP and *E. coli* RNAP in utilizing *rrnO*-P1P2 template

Transcription reactions were carried out in transcription buffer containing 150 mM NaCl. 0.1 pmoles of DNA templates and 0.5 pmoles *B. subtilis* RNAP were used in the reactions. Reactions were initiated by addition of RNAP, incubated (37^0 C) for 16 min, and terminated with stop solution (2.5 M NH_4OAc, 20 mM EDTA, and 100 µg/ml carrier plasmid DNA). The molar ratios of transcripts were calculated by correcting for the number of UMP residues incorporated into each run-off transcripts. (A) transcriptions of P1 and P2 by *B. subtilis* RNAP and *E. coli* RNAP. Lane 1: *E. coli* RNAP; lane 2: *B. subtilis* RNAP. b) and c) comparison of the efficiencies of the *B. subtilis* RNAP and *E. coli* RNAP in utilizing the P1 and P2 promoters on the *rrnO*-P1P2 template. *B. subtilis* RNAP efficiency were equal to 1.







APPENDIX

Tryptose Blood Agar Base (TBAB) (1 liter)

5g Bacto-agar

33g Tryptose Blood Agar Base (TBAB)

Bring to 1000 ml with deionized water and autoclave. After autoclaving, add

0.2 ml 0.1 M $MnCl_2$

5 ml 1.0 M $MgSO_4$

10X Concentrated Spizizen Salts (2 liters)

In 1.5 liters of deionized water, add in the following in order, making sure each salt dissolves before adding the next one.

1) 40 g AMMONIUM SULFATE ($(NH_4)_2SO_4$)
2) 120 g POTASSIUM PHOSPHATE MONOBASIC (KH_2PO_4)
3) 280 g POTASSIUM PHOSPHATE DIBASIC (K_2HPO_4)
4) 20 g SODIUM CITRATE ($Na_3C_6H_5O_7$-$2H_20$)
5) 49 g MAGNESIUM SULFATE ($MgSO_4$)

Bring to two liters with deionized water

20X Concentrated sodium saline citrate (2 liters)

351g NaCl

176.4g SODIUM CITRATE

Bring to two liters with deionized water and pH to 7.0

10X TE

100 mM Tris-HCl, pH 8.0

10 mM EDTA, pH 8.0

10X TBE

108g Tris base

55g Boric acid

40 ml 0.5 M EDTA, pH 8.0

Bring to one liter with deionized water

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