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DIFFERENTIAL RESPONSE OF *BACILLUS SUBTILIS* RIBOSOMAL RNA OPERONS TO NUTRITIONAL STRESS

BY

WALIED SAMARRAI

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

1996

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Abstract

DIFFERENTIAL RESPONSE OF *BACILLUS SUBTILIS* RIBOSOMAL RNA OPERONS TO NUTRITIONAL STRESS

by

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The expression of ribosomal RNA operons (*rrn*) of *B. subtilis* during fast and slow growth and the intrinsic promoter strength of P1 and P2 were evaluated by placing promoter bearing fragments from *rrnO, rrnJ, rrnB, rrnD* and a *veg* control at the *amyE* locus using pDH32/pDG268 containing a *spoVG-lacZ* fusion-gene with two large regions of the *amyE* gene. The levels of *rrn* operons expression as measured by β-galactosidase, dot blot and primer extension assays revealed definite heterogeneity; namely, strong (*rrnO*), intermediate (*rrnJ*), and weak (*rrnB, rrnD*). The intrinsic promoter strengths were determined from the relative abundance of RNA transcripts using primer extension analysis. The P2/P1 ratios were 9.10 and 12.5 for *rrnO* and *rrnJ,* respectively. Only P2 transcripts were detected for the weak promoters of *rrnD* and *rrnB* while *veg* produced only P1 transcripts. Similarly, the response to stringent control during amino acid starvation induced by serine hydroxmate (SH) or carbon limitation induced by α-methyl glucoside (αMG) was variable; *rrnO* and *rrnJ* showed a strong effect (8-15 fold

decrease), *rrnD* and *rrnB* barely responded (1.3-fold decrease) and *veg* remained unchanged. Promoter elements P1-P2, P1 or P2 of *rrnO* and *rrnJ* lacking Upstream Activating Sequences (UAS) were synthesized using PCR, cloned in pDG268 and integrated into three genetic backgrounds (*relA*$^{+}$, *relA*$^{-}$ and *relA*$^{(S)}$; the latter is a suppressor that responds only to carbon limitation) revealed the importance of the upstream region in the maximal expression levels. Only the strong P2 elements responded to carbon-source limitation in the wild-type and the suppressor cells, while the weak P1 elements continued to function in all strains. When a *relA*$^{+}$ strain was treated with SH, both P1 and P2 responded by showing a dramatic decrease in the synthesis of *lacZ*-mRNA and a concomitant accumulation of (p)ppGpp. In the *relA*$^{-}$ and *relA*$^{(S)}$ backgrounds RNA synthesis is relaxed. During rifampin challenge, (p)ppGpp accumulated with a concomitant decrease of GTP in all cell line. Mutants in the *rpoB* gene did not accumulate guanosine polyphosphates. Finally, growth rate regulation of *rrnO* was abolished in a double mutant (*relA*$^{-}$, *rpoB*) and not in the single mutant strains (either *relA*$^{-}$ or *rpoB*). We conclude that in *B. subtilis*, (p)ppGpp is the inhibitor of rRNA synthesis even in the absences of amino acid starvation and is involved in growth-rate regulation.

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Abbreviations

Serine hydroxmate	SH
α-methyl glucoside	αMG
RNA polymerase	RNAP
ribosomal RNA	*rrn*
Guanosine 3'-diphosphate 5'-diphosphate	ppGpp
Guanosine 3'-diphosphate 5'-triphosphate	pppGpp
(p)ppGpp synthetase I	PSI
(p)ppGpp synthetase II	PSII
Chloramphenicol acetyl-transferase	CAT
RNA tandem promoters	P1P2
RNA upstream individual promoter	P1
RNA downstream individual promoter	P2
Minimal medium	MM
Veal infusion yeast extracts	VY
Rifampin resistance	rif^R
Chloramphenicol resistance	CM^R
Wild type relA gene	*relA*$^+$
Relaxed relA gene	*relA*$^-$
relA suppressor gene	*relA*^{S}
Amylase gene	*amyE*

Introduction

A. Organization of stable RNA genes in *Bacillus subtilis* .

Ribosomal operons are unique among procaryotic genes because of their central role in metabolism and because of their multiplicity, which gives rise to genetic variability. Most of what is known about ribosomal genes comes from work done in *Escherichia coli.* Although many of the regulatory features observed in *E. coli* are likely to be similar in *Bacillus subtilis*, both the control and genomic arrangement of *B. subtilis* ribosomal genes are expected to differ because of the sporulation and germination functions that are not part of the *E. coli* life cycle (1). The number of ribosomal RNA gene copies in different organisms varies greatly; bacteria possess from one to 10 *rrn* operons per genome, while there are hundreds or even thousands of ribosomal transcription units per eukaryotic genome (2). In the gram-negative enterobacterium *E. coli,* there are seven ribosomal RNA operons (*rrn*) (3, 4). The gram-positive *B. subtilis* genome contains 10 rRNA operons, [and there is a high clustering of tRNA genes (5, 6, 7, 8, 9)]. Nine of the ten rRNA gene sets are located between origin O and 70 on the `qgenomic map (Fig. 1). Two sets of closely spaced tandem clusters are present. The first consists of two gene sets (*rrnJ*, *rrnW*), and the second consists of three gene sets *(rrnI rrnH, rrnG)* (8). The ribosomal RNA gene sets of *B. subtilis* have been shown to be transcribed in the order 16S, 23S, and 5S (3) exclusively from the H strand (11). Each gene set represents a distinct operon transcribed from

unique tandem promoters (P1, P2), as in *E.coli* (4), and has a presequence P2-16S-spacer-23S genes-5S genes- terminator region (1,12) (Fig 2). The organization of tRNA genes in *B. subtilis* is quite different from what is observed in *E. coli,* where the majority of tRNA genes are cotranscribed with rRNA genes (7).

Many features within the promoter regions of stable RNA genes in *E. coli* have been studied:

1) Upstream activating sequences (UAS) in the region of -98 to -40 increase promoter strength (13,14), and have been best studied in the *E. coli rrnB* operon which is activated 20-30- fold *in vivo* by this sequence(13). The UAS contains binding sites for the factor-dependent fis protein which stimulates *rrn* expression 10-20 fold upon binding (15). Ribosomal RNA operons have three consensus binding sites upstream of the P1 promoter, although actual binding has only been demonstrated for *rrnB.* In addition, there is an AT rich factor-independent subregion which is responsible for the additional 2-4 fold activation by UAS (16).

2)Tandem promoters, P1 and P2, consist of two distinct -35 and -10 regions separated by about 110 to 120 bp (14).

3) The discriminator region which spans positions -7 to -1 is characteristically GC-rich (5'GCGCC-C3') and is highly conserved for stable RNA promoters (12,17).

4)Leader regions specifying *rrn* antiterminators are localized between P2 and the beginning of 16S (18,19,20).

In contrast to *E. coli*, the promoters of the rRNA operons of *B. subtilis* are more heterogeneous in both sequence and function. A number of unique features have been observed and are summarized in table 1.

1) The ribosomal promoters differ significantly from one another in levels of expression: the strongest being *rrnO* and *rrnW;* intermediate *rrnA*, *rrnJ*, and *rrnE*, and the weakest, *rrnD* and *rrnB* (1).

2)The nucleotide sequence of promoter P1 of *rrnO* is unique when compared to the other 6 promoters that have been sequenced (Table 1). On the basis of S1 mapping experiments, the P2 promoter is more effectively used while transcription is rarely initiated off of P1 (12, 21).

3) The 3'-end of the leader sequence in *rrnO*, *rrnD* and *rrnB* is 65bp longer than what is observed in the other operons and allows for the formation of complex stable secondary structure (12, 22, 23).

4) The P2 promoters of *rrnB* and *rrnJ* are growth rate regulated and are the more transcriptionally active when assayed in *E. coli* (1, 24).

5) Only two of the ten rRNA operons contain internal tRNA genes in the *16S-23S* intergenic space (1, 12, 22, 9). Operons *rrnO* and *rrnA* have $tRNA^{ILe}$ and $tRNA^{ALa}$ determinants in the spacer region (Fig. 2) (1).

The presence of multiple *rrn* operons is the primary mechanism used by cells in order to maintain the necessary amount of rRNA within them. It has been shown previously that deletion of one of the seven *rrn* operons in *E. coli* or two of the ten *rrn* operons in *B. subtilis* (*rrnG* and *rrnW*) (25, 26) had no observable effects on either cell growth rates or cellular physiology, suggesting that neither organism requires the full complement of *rrn* operons. In contrast to the wild type strain the growth rate of *E. coli* started to decrease when three *rrns* were deleted and with four operons inactivated the growth rate was decreased further. Similarly, there was no significant alteration in ribosome concentration until the third *rrn* was inactivated. This level decreased further when four *rrn* operons were deleted (27).

Control mechanisms in prokaryotes:

Bacteria are known to adapt their growth rates to their nutritional conditions and to other environmental signals like salt stress, oxygen limitation or heat stress. Changes from slow to fast growth rates are achieved by a rapid increase in the number of ribosomes per cell. Under rapid growth conditions, the fraction of rRNA and tRNA constitutes more than 95% of the total cellular RNA. Many different regulatory mechanisms which involve both *cis* and *trans* acting components participate in the production of RNA. The synthesis of stable RNA in bacteria is regulated over a wide range of growth conditions. Three transcriptional

regulatory mechanism exist in bacterial system, stringent control, RNA polymerase partition pattern and growth rate dependent control.

Stringent control

Adaptation to starvation conditions is a basic phenomenon in bacteria. The cellular response to amino acid starvation entails numerous regulatory cellular activities. This has come to be known as the stringent response. This response could be relaxed by inactivating a genetic determinant that has been mapped to a single RNA control locus called *relA* (28,29). The starvation response in the cell was shown by depriving cells of an amino acid, by inactivating an aminoacyl-tRNA synthetase or by using a glucose uptake inhibitor. Cells under stringent conditions show accumulation of guanosine nucleotides guanosine 3'-diphosphate 5'-triphosphate (ppGpp) and guanosine 3'-diphosphate 5'-triphosphate (pppGpp). The accumulation of the phosphorylated compounds is accompanied by a reduction in the rate of total RNA accumulation. This is due largely to a dramatic (10-20 fold) decrease in the accumulation of stable RNA (29). The (p)ppGpp was also shown to accumulate in *B. subtilis* and *E. coli* upon the inhibition of protein synthesis in growing cells (30). This accumulation was only observed when using protein synthesis inhibitors that act at the ribosomal level (30).

In both *E. coli* and *B. subtilis* the accumulation of the guanine nucleotide (p)ppGpp is correlated with *relA* gene function during the stringent response (29,

31, 32). The *relA* mechanism involves the activation of (p)ppGpp synthetase (PSI) by high ratios of uncharged/charged tRNAs (33). The (p)ppGpp synthetase activity requirements (ribosomes, mRNA and codon specified uncharged tRNA bound in the A site) neatly account for the physiological features of (p)ppGpp synthesis (29). In *E. coli, relA* mutants are unable to respond to amino acid limitation. However they continue to respond to carbon source limitation (34). The response of *E. coli* to carbon source shift-down which is accompanied by (p)ppGpp accumulation is mediated via a *spoT* gene-dependent mechanism (34). Although the *spoT* gene product is responsible for (p)ppGpp degradation, it also appears to function as a second (p)ppGpp synthetase (PSII) (35). The activity of the *spoT* gene product is stimulated by Mn^{2+} and is inactivated by treatment with chelating agents like 1,10-phenanthorline (29). Other genes have been found in *E. coli* that are important in understanding the stringent response and the metabolism of the (p)ppGpp nucleotides. Among these genes are *relB, relC, relX* and *rpoB* (29).

The accumulation of the polyphosphorylated nucleotides in *B. subtilis* has been examined in relation to the function of the *relA* gene. It was found that during inhibition of isoleucine activation by O-methylthreonine, wild type *B. subtilis* shows accumulation of (p)ppGpp. The accumulation of (p)ppGpp was also noticed with carbon source down shift elicited by inhibiting glucose uptake. The *relA* mutant did not accumulate any (p)ppGpp under any of the above conditions (32, 38).

An activity similar to what is observed for the *E. coli spoT* gene product has also been reported in *B. subtilis* (37). As with the *spoT* product of *E. coli* activity can be inhibited by the chelating agent 1,10-phenanthroline. The accumulation of (p)ppGpp can also occur in *B. subtilis* during the inhibition of the *spoT* gene -like product function (37). Spontaneous mutants of *B. subtilis* resistant to thiostrepton *(tsp)* also exhibit relaxed synthesis of RNA when starved for required amino acids. Intact cells of *tsp* mutants cannot synthesize the regulatory nucleotide (p)ppGpp during amino acid starvation conditions (39). The phenotype of the *tsp* mutant has been mapped to the *relC* locus at a map position adjacent to *spoOH.* This is different from the *relA* position which has been mapped to a region between *aroD* and *leu* (274^0) (38, 40). The *relC* strain is missing LII from the 50S ribosome subunit (39). The mapping data shows that the *B. subtilis relA* gene is quite distant from the major ribosomal gene cluster (38). This is similar to the map of *E.coli*, where *relA* maps at 59', the major ribosomal protein gene cluster is at 72', and *rplK* (LII) is found at 88.5' (41). The significance of the *B. subtilis relA* map position is unclear, although it is close to several *rec*, *dna* and *div* genes. The *E. coli relA* gene is also found near various *rec* and *dna* genes (41). Another mutation in the *relG* gene can produce a relaxed *B. subtilis* strain. This mutation could be responsible for a relaxed response to glucose depravation (42). The *relG* mutant can still accumulate (p)ppGpp in response to amino acids

starvation (albeit to a lesser extent than its *relA*$^{+}$ parent), but not after glucose depravation (42).

It was suggested by Cashel that (p)ppGpp might be the pleiotropic effector responsible for both regulating the synthesis of stable RNA and underling all the other physiological changes characteristic of the stringent response. However, the mechanism by which (p)ppGpp controls ribosomal RNA synthesis has yet to be elucidated (29). Hernandez and Bremer (36), demonstrated that the *rrnB P1-lacZ* fusion of *E. coli* showed an exponential decrease in activity as the cytoplasmic concentration of (p)ppGpp increased due to growth in poor medium or by using a strain with *spoT* allele (36). It has also been shown in *E. coli* that the elevation of ppGpp levels under nutritionally adequate conditions mimicked the slowness in growth, the inhibition in protein synthesis and the overall inhibition of stable RNA accumulation (all of which is classically associated with elevated ppGpp during the stringent response) (43, 44).

Reports concerning the response of individual promoters to stringent conditions in *E. coli* demonstrated that the upstream promoter (P1) is subject to both stringent control and growth rate control (4). The downstream promoter (P2) has been shown to be relatively insensitive to these regulatory mechanisms, behaving like a weak constitutive promoter (29, 46). Recent studies in *E. coli* have revealed that the activity of P1 interferes with P2 expression. When the activity of P2 is measured in separate constructs of P1, its activity was as high as 70% of the

signal obtained from P1 and it was subject to stringent control like the P1 promoter (47).

The role of RNA polymerase

The question whether or not RNA polymerase is the target of (p)ppGpp action has been investigated. A study in *B. subtilis* showed that RNA polymerase (RNAP) is directly involved in (p)ppGpp metabolism (48). When exponentially growing cells were treated with rifampin or liparmycin, both of which inhibit the initiation of ribonucleic acid synthesis, (p)ppGpp accumulated in the wild type *relA*$^+$ as well as in both *relA*$^-$ and *relC*$^-$ mutants. The authors suggested that there is a second mechanism responsible for (p)ppGpp accumulation, a reaction which is dependent upon free or initiating RNA polymerase molecules and independent of the normal ribosome-mediated process (48).

Studies in *E. coli* using a plasmid, which carries the *E. coli relA* encoding a ppGpp synthetase gene under the control of the *lacUV5* promoter showed that the expression of β-galactosidase from an *rrnB* P1 promoter is inhibited by ppGpp. This suggested that ppGpp is a specific inhibitor of rRNA synthesis, even in the absence of amino acid starvation (35). Following continued incubation at 37°C, a particular mutant strain was isolated. This mutant was fast growing and had acquired partial resistance to ppGpp inhibition of rRNA synthesis. Phage P1 transduction experiments showed that this mutant has mutation cotransduct

with *rpoB*, the gene encoding the β-subunit of RNAP. These results indicate that RNAP is involved as the target of ppGpp action (35).

It was also found in *E. coli* that ppGpp affects the binding of RNA polymerase to stable RNA promoter sequences in a filter binding assay (49). Another Study showed the involvement of RNAP with ppGpp and RNA synthesis (45). In this study the rates of synthesis for DNA, rRNA, bulk mRNA, protein and RNA polymerase β- and β'- subunits as function of growth rate in a wild-type *E. coli* strain, and in the Δ*relA* Δ*spoT* mutant which does not produce ppGpp. The authors (45) concluded that the rate of stable in RNA synthesis per amount of protein depends upon three factors: RNAP concentration, RNAP activity, and the distribution of active RNAP between stable and mRNA genes, as determined by measuring as the stable RNA synthesis rate / total RNA synthesis rate, r_s/r_t. In the wild-type strains, all three factors increase along with growth rate. In the ppGpp-deficient strains, only RNA polymerase synthesis and activity, but not r_s/r_t, increased with growth rate. Thus, adjustments of RNA stable / RNA total require ppGpp. In addition, in the absence of ppGpp, the synthesis of both rRNA and bulk mRNA varied in direct proportion to the concentration of active RNA polymerase. This is in contrast to the wild-type strain, where only rRNA synthesis increased with growth rate, while mRNA synthesis remained constant (45).

Growth rate control

Bacterial cells are able to constantly monitor the environment so that enough but not too many ribosomes are synthesized. How the cell senses the state of the nutritional environment with respect to the availability of energy and materials, and how it adjusts the rate of ribosome biosynthsis are critical questions that have been extensively studied. In the literature, two models have been proposed, the direct effector model and the ribosome feedback regulation model.

The direct effector model suggests that ppGpp is a direct effector which interacts with either RNA polymerase by sequestering it in the elongation complex. or by separating the RNAP into a (p)ppGpp-bound and free (unbound) forms (36). The ribosomal feed back regulation model suggest that there is an equilibrium between those ribosomes that are actively translating and those that are free. If the equilibrium shifts so that the pool of free ribosomes increases, further production of ribosomes would be repressed by inhibiting rRNA transcription. On the other hand, if the equilibrium shifts so that the pool of free ribosomes is depleted, rRNA synthesis would be derepressed, resulting in an increased rate of ribosome biosynthesis (4).

Studies in *E. coli* examined the effects of extra, plasmid-born rRNA genes on the synthesis rate of rRNA by measuring the fraction of total RNA synthesis that is rRNA and tRNA, the cytoplasmic concentration of (ppGpp), and the

absolute rate of RNA and protein synthesis. These experiments were carried out in different growth media using two different strains of *E. coli* (one harboring the entire *rrnB* transcriptional unit and the other harboring a defective *rrnB* transcriptional unit). These studies show that extra intact, but not extra defective, plasmid-borne *rrn* genes caused the level of ppGpp to increase. The ratio of stable RNA to total RNA was increased with either intact or defective *rrn* genes as a function of ppGpp levels. Finally, the rRNA synthesis rate */rrn* gene was reduced in the presence of extra *rrn* genes. This observed reduction in gene activity was greater with intact than with defective *rrn* genes. These results are consistent with the ppGpp hypothesis of rRNA control but not with a feedback effector role of translating ribosomes (53).

Studies supporting the feedback inhibition model showed that inactivating four rRNA operons in *E. coli* will result in an increase in *rrn* expression of the remaining operons of such magnitude to compensate for the number of operons inactivated. The increase in expression occurred in the absence of changes in the intracellular concentration of ppGpp. Therefor the authors (27) suggested that ppGpp is not likely to be involved in the growth rate regulation of these cells (27).

The regulation of rRNA levels promoted by growth rate control was studied in *B. subtilis* (55, 1). The *rrnB, rrnJ* of *B. subtilis* and *rrnB* of *E. coli* were fused to the reporter gene chloramphenicol acetyl-transferase (CAT). The level of CAT expression in *E. coli* showed growth rate dependence from either *E. coli*

or *B. subtilis* tandem promoters. The downstream promoter (P2) of the tandem *Bacillus* pair was shown to be growth rate regulated and transcriptionally more active relative to the upstream promoter (P1) (1, 24). In *E. coli,* it was found that *rrnB* has a sequence between -20 and -51 of the P1 promoter that endows growth rate-dependant control. In addition, the upstream promoter (P1) showed growth rate regulation and is transcriptionlly more active relative to the down stream promoter (P2) which appears to be a weak and constitutive promoter (24).

Research objectives

The objectives of this study is essentially three fold:

Frst, to establish whether or not all *B. subtilis* ribosomal RNA operons are expressed and regulated similarly or whether the chromosomal location and the sequence heterogeneities observed in the control regions cause differential promoter activities under different physiological conditions.

Second, to determine the patterns of relative expression of the tandem and the individual promoter elements of two *B. subtilis* rRNA operons *rrnO* and *rrnJ*, under different nutritional stress conditions.

Third, to determine in *B. subtilis* the role of the *relA* gene and RNAP in both (p)ppGpp metabolism and in growth rate regulation .

MATERIALS AND METHODS

Bacterial strains, plasmids.

The *B. subtilis* strains used are listed in Table 2A and their phenotypes were described previously (40). The three strains IS58, IS56 and L3 are isogeneic (*trpC2, lys-3*) differing only with respect to the *relA* gene. Phenotypically they differ by resistance or sensitivity to 15 mM AT and the associated amino acid sensitivity in complex medium (40). The phenylalanine requirement is found for IS56, L3 as well strains with Tn*917* insertions in the *relA* region (Table 3) (40). Strain 1A308 a rif^R mutant the *rpoB18* gene (56) was used as a source of transforming DNA for strain constructions (Table 2A).

A single copy integration plasmid pDH32 /pDG268 was used (Fig. 3) (56, 57). This plasmid carries *a spoVG-lacZ* fusion with *B. subtilis* translation signals (RBS), a multi cloning site sequence, and two large regions of the *amyE* gene (*amyE* front and the *amyE* back) (Fig. 4a). This plasmid can integrate into a heterologous locus the *amyE* gene, at 25° on the map. Integration at this locus yields $amyE^-$ clones by a double cross over mechanism (58), or $amyE^+$ clones by a Campbell-type insertion at either *amyE* front or *amyE* back region (Fig. 4b). This mechanism was verified by Southern blot analysis, using a ^{32}P labeled 1.3 kb *amyE* back fragment was used as a probe. The intact promoter bearing fragments from *rrnO* or *veg* were cloned in to pDH32 by C. Stewart. The *veg* gene served as control (71). Intact promoter bearing fragments from *rrnB, rrnD* and *rrnJ* were

cloned in pDG268 by A. M. White. The source and the size of these fragments are as following: *rrnB* in pAWR123 was obtained as 1.8Kb *EcoRI* fragment from pGS227 given to us by K. bott (24, 33). The *rrnD* fragment in pAWR116 was rescued by E. Jarvis as 2.2 *HindIII* fragment in plasmid pJR421 (23). The *rrnJ* in pWR113 was obtained as 1.7Kb PstI fragment in pGR151(1). The *rrnO* was obtained from the E19 fragment in pMS102 cut by EcoRI (72).

These promoter bearing fragments were fused to the *lacZ* reporter gene of the pDG268 plasmid. The *rrn-lacZ* fusion constructs of *rrnO*, *rrnJ, rrnD, rrnB,* and *veg* gene control were linearized with *PstI,* and transformed into IS58 ($trpC_2$, lys-$_3$) as well as into other strains. The chloromphencol (CM) sensitive strains transformed with the constructed plasmid and the Cm^R clones which were also blue on the X-gal. plates were selected. The $amyE^+$ phenotype was detected on starch-TBAB agar plates (3.3% Tryptose Blood Agar Base, 0.5% Bacto Agar, and 0.2 ml of 0.1 M $MnCl_2$, 50 mM $MgSO_4$ per liter, and Potato starch 1%). The strains were also grown on starch plates. The starch plates were flooded with I_2-KI to see if they can hydrolyze starch ($amyE^+$) or not ($amyE^-$).

To study the functions of different *rrn* promoter elements, the fragment bearing promoter P1 and P2 were also fused to the *lacZ* reporter gene of the plasmid pDG268. These promoter fragments were constructed from PCR by David Liu for the two *rrn* operons *rrnO* and *rrnJ* (Fig. 2). These plasmids were transformed into the strain of interest (IS58, IS56, L3).

Preparation of competent cells

B. subtilis were made competent using a previously published method (59), as modified by (60). An overnight, 10 ml VY culture was pelleted in a clinical centrifuge and resuspended in 5 ml of Spizizen minimal medium I (1x Spizizen salts, 0.5% glucose, 0.02% casamino acids (Difco), 0.1% yeast extract, 0.8% L-arginine, 100 µg/ml of required amino acids). This culture was used to inoculate a sidearm flask containing 20 ml of the same medium to a Klett of 30-35. After growth at 37°C with shaking for 4.5 hours, the culture was, diluted 1 to 10 with Spizizen minimal medium II (1x Spizizen salts, 0.5% glucose, 0.01% casamino acids, 0.05% yeast extract, 2.5 mM $MgCl_2$, 1mM $CaCl_2$, 0.05 mM spermine tetrahydrochloride, 5 µg/ml of required amino acids) and grown for 90 minutes. At this time the culture was either used in a transformation assay or frozen for storage. Freezing was done by pelleting cells at room temperature in sterile centrifuge bottles, resuspending them in 1/10th volume of minimal medium II containg 5% glycerol, and quick freezing 2 ml aliquots in a dry ice-ethanol bath. frozen cells were reconstituted with minimal medium II after quick thawing at 65°C. Plasmid DNA (5-10 µg) or chromosomal DNA (0.2 to 2 µg) in 0.1 ml was mixed with 0.9 ml of fresh or reconstituted competent cells and incubated on a culture roller for 30 minutes at 37°C. DNAase I (Cooper Biomedical) was added (0.1 ml of a 100 µg/ml solution in 0.25 M $MgSO_4$) and these tubes were incubated for an

additional 10 minutes. Cells were plated at appropriate dilutions onto plates containing Cm or selective minimal plates. Plates were incubated at 37°C for 24 to 48 hrs.

Chromosomal DNA Isolation

B. subtilis chromosomal DNA was prepared by pelleting cells from an overnight VY culture (2x 500 ml), resuspending in 0.15 M NaCl. 0.1 M EDTA pH8.0 (4 ml per gram wet weight of cells), and incubation with lysozyme (7-10 mg) at 37°C for 1-1.5 hrs. Sodium dodecyl sulfate was added to a 2.5% final concentration and the lysate heated at 60°C for 10 minutes. DNA was extracted by the procedure of Marmur (66) as modified by Rudner et al (60). Concentration of purified DNA was estimated by its absorbance at 260 nm. Typically, 1 mg of DNA was recovered per gram wet weight of cells. Stock DNA was diluted to 100 µg/ml with TE and used as a working solution.

Growth conditions and labeling intact cells.

Liquid cultures of *B. subtilis* were grown in complex medium (VY), composed of 2.5% veal infusion (Difco)- and 0.5% yeast extract (Difco). The basal medium (MM) for all growth rate experiments was Spizizen minimal salts (34) supplemented with 50 µg/ml of L- tryptophan and 100 µg/ml of L-lysine or L-histidine. To achieve different growth rates the basal, medium was supplemented

with the following: MM1-0.5% glucose, 1% Na-glutamate; MM2-1% Na-succinate, 0.05% yeast extract, 0.02% vitamin-free casamino acids; MM3-1% Na-acetate, 0.05% yeast extract, 0.02% vitamin-free casamino acids. Overnight VY or MM1 cultures of the various strains containing integrated *rrn-lacZ* fusions were centrifuged, washed and diluted 1 in 25 with fresh, prewarmed media of the three types of media and then shaken at 37^0C in 250 ml, sidearm flasks. Growth was followed using a Klett-Summerson spectrophotometer equipped with a red filter. During logarithmic growth at a Klett reading range of 20-250, samples (2 X1.0 ml) for β-galactosidase assays and (2X 2.0- 4.0 ml.) for RNA slot blots were withdrawn and processed. The stringent response or transcriptional inhibition was induced to cultures grown in MM1 by the addition of serine hydroxamate (SH; 2 mg/ ml), α-methylglucoside (αMG; 1%) or rifampin (10 μg/ml) at a Klett reading of 100. Similarly, samples were withdrawn at 0, 30, 60 and 90 minutes in the case of SH or αMG and at 0, 5, 10 and 20 minute following rifampin addition.

A low-phosphate Tris-glucose medium described previously (32, 38, 40) containing: 0.1M Tris-HCl (pH 7.4), 0.1 mM KH_2PO_4, sodium citrate (0.4 mg/ml), $MgSO_4 7H_2O$ (0.2 mg/ml), $(NH_4)_2SO_4$ (1 mg/ml), $FeCl_3$ (0.32 μg/ml), glucose (0.5%) was used to labele the pool of nucleotides. The following amino acids were added: lysine, proline, glycine, alanine, glutamic acid, aspartic acid, and arginine, all at 100 μg/ml; and cysteine, methionine, tyrosine, tryptophan, and phenylalanine, all at 40 μg/ml. Cultures were labeled with {^{32}P}-phosphoric acid

(50-100 μCi/ml) for one generation (1h.), and followed by treatments with either SH, αMG or rifampin as described above. Samples of 100 μl were withdrawn at 0, 5, 10, and 15 min were mixed with 13 M formic acid and frozen.

Measurement of phosphorylated guanosine nucleotides

The labeled samples were centrifuged in an Eppendorf .microcentrifuge for 5 min. Supernatant (10 μl) were applied to polyethyleneimine-cellulose (PEI) plates (Brinkmann Instruments) for separation by thin-layer chromatography of the phosphorylated guanosine nucleotides in 1.5 M KH_2PO_4 (32, 38, 40). Radioactively labeled nucleotides were located by autoradiography. The relative concentrations of pppGpp, ppGpp and GTP determined by densitometry using a Zeineth soft scanning densitometer (Model SL-DNA; Biomed Instruments).

β-galactosidase assays

Strains with integrated pPW4, pAWR118, pAWR116, pAWR123, and pPW810 plasmid were assayed for β-galactosidase activity by the method of Miller (61). One milliliter cell samples from cultures growing in VY or MM1 medium were taken at various Klett readings. After pelleting cells in a clinical centrifuge, the cells were resuspended in 0.9 ml of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCL, 1 mM $MgSO_4$, 50 mM β-mercatoethanol, pH 7.0). Incubation with 250 μg /ml lysozyme for 10 minute at 37°C was followed by vortexing in the presence

of 0.1% Triton X-100. Samples (0.5 ml) of this lysate was added to 0.5 ml Z buffer. Reactions were initiated by the addition of 0.2 ml of o-nitrophenyl β-galactosidase (ONPG, 4 mg/ml in 100 mM phosphate pH 7.0 and stopped when sufficient yellow color had developed by the addition of 0.5 ml of 1 M Na_2CO_3. Cell debris was pelleted in a clinical centrifuge before reading the A_{420} and A_{550}. Miller units were calculated using the following formula:

$$1 \text{ unit} = 1000X \frac{(A_{420} - A_{550})}{v.\ t.\ A_{600}}$$

where v= volume of lysate used and t= incubation time of reaction.

100 klett reading = 0.5 OD

RNA isolation

RNA was isolated using a modification of the procedure described for *E. coli* (62) from 2.0- 4.0 ml samples removed from cultures grown only in minimal medium (MM1, 2 or 3). Cells were centrifuged and immediately resuspended in 2.0 ml freshly made lysis buffer mix #1: [2.0 mg lysozyme /ml, 2 U/ml of RNase-free DNase (RQ1TM, Promega) 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA] and placed on ice for 10 min. This was followed by the addition of 0.5 ml of hot lysis buffer mix #2: [1M Nacl, 50 mM EDTA (pH 8.0), 2.5% SDS]. Samples are placed in boiling water for 30 sec. Following phenol extraction and ethanol precipitation,

the RNA was dissolved in sterile water and the intactness of the preparations was judged by visualization of ribosomal RNAs bands on ethidium-bromide stained agarose gels.

Dot blots assay

The levels of the *LacZ*-mRNA in total RNA were determined by hybridization to a probe specific for the *lacZ* message by dot-blot analysis (63). 5-10 μg/ml samples of total RNA were loaded onto either nitrocellulose (S&S, Schleicher &Schuell, Keene ,NH, BA-S NC) or Nytran nylon (S&S) inserted in a dot blot apparatus made by S&S. The filters were baked, prehybridized, hybridized, and washed, as previously described (63). The source of the specific *lacZ* probe was pDEB1 (64), a parent plasmid for integrative *lacZ*-fusions, linearized with *Hind*III and labeled with the random primer extension kit as directed by the supplier (USB, Cleveland, OH) using [α-^{32}P]dCTP. The specific activity was routinely 1-3 X 10^8 CPm/μg. The filters were exposed for 17 h to X-omat AR film (Eastmen Kodak Co., Rochester, NY) with intensifying screens at -70^{0}C. Autoradiographed films were scanned for hybridization intensity using the Zeineth soft scanning densitometer.

Primer Extension analysis

Primer extension reactions were performed with modifications (63) of a procedure described for *E. coli* (47, 62). Total RNA from 2 ml of culture. (75 μg)

was mixed with an excess of purified 5' labeled primer (8-10 ng, see below) in the presence of hybridization buffer (0.1 M KCl, 0.05 M Tris-HCl, pH 8.3). This mixture was heated for 1' at 90^{0}C for denaturation, followed by 2' at 60^0 C and then placed on ice for 15' to allow anneal. 5XRT buffer (0.25 M Tris-HCl pH7.9, 0.2 M KCl, 0.036 M Mg Acetate, 0.01 M DTT, 1 mM dNTPs and 2u/µl RN-asin (Promega) was added to a final concentration of 1X. The primer extension reaction was initiated by the addition of 20U of AMV reverse transcriptase (Molecular Genetic Resources) The reaction mixture was then incubated for 1 hr at 45^0 C and stopped by the addition of an equal volume of dye mix (0.1%bromophenol, 0.1% xylene cyanol in deionized formamide). The mixture was heated for 3' at 100^0 C, and the primer extensions products were separated by electrophoresis on a 6%acrylamide-urea sequencing gel and visualized by autoradiography. Quantitation of primer extension products was done by densitometry using the Zeineth soft scanning densitometer or a PDI (protein & DNA imageWare systems. The primers used were: 5'- TGC AGG CCC TAG TTT GAC TGA CTA C 3' complimentary to the unique sequence of *rrnO* at -256 to -221; (12) and 5'- TCA GTA ACT TCC ACA GTA GTT CAC CAC CTT 3' complimentary to the *spo*VG-*lac*Z junction between the *SalI* and the *BamHI* sites at 3771 to 3741 of pDH32 (58). The primers were first purified by gel filtration using a 10 ml fine Sephadex G25X column, and the peak fraction concentrated by evaporation. 100 ng of the purified primers were then end-labeled with 40 µCi of [γ-^{32}P-ATP] in

hybridization solution (5X kinase buffer, 0.5 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$), 50 mM DTT and 5-10 units of T4 polynucleotide kinase (Boerhringer Mannheim). The reaction was incubated at 37^{0}C for 30 min and then purified through a Quick Spin G-25 Sephadex column and stored at a concentration of 70-100 µg/ml.

DNA sequencing

The plasmids for double strand DNA sequencing were purified by using the QIAprep Spin Plasmid Kit (QIAGEN). The procedure for dideoxynucleotide DNA sequencing was carried out according to the instruction provided with the USB DNA sequencing kit.

Results and Discussion

I- Differential expression of *four rrn* promoters at a heterologous-*amyE* locus:

Early studies in this laboratory with a *rrn-lacZ* fusion (pWR112), containing *E. coli* translational signals, inserted via homologous recombination into 7 out of 10 resident *rrn* promoters of *B. subtilis* revealed that they are not equivalent in their expression levels (1). The expression levels were correlated with their genomic location and the promoters were grouped into strong (*rrnO, rrnW*), intermediate (*rrnA, rrnJ, rrnE*) and weak (*rrnD, rrnB*) promoters (1). They also responded to growth-rate regulation by a 2-4 fold increase in the levels of β-

galactosidase after a shift-up from minimal medium (MM1) to complex medium 1. To evaluate intrinsic promoter strength of a representative number of ribosomal RNA genes sets, five fragments (*rrnO, rrnJ, rrnD, rrnB*, and *veg* control) were fused to the *spovG-lacZ* fusion gene contained on plasmids pDH32 or pDG268 and integrated stably into the chromosome of *B. subtilis* via double recombination with the *amyE* gene (57, 58) (Fig. 4 and Table 1A). As described in Material and Methods, these five fusions were linearized with *PstI*, transformed into a *relA*$^{+}$ background (strain IS58). Cm^{R} transformants were selected which were *amyE*$^{-}$ and had a blue phenotype on minimal agar plates containing X-gal. In order to determine extent of the functional heterogeneity among the four rRNA operons, we used, β-galactosidase assays, RNA dot blot and primer extension assays.

A. β- galactosidase assays

To determine the relative promoter strength at the translation level for the four operons, the constructed integrant strains were grown on minimal salts containing glucose/glutamate (MM1) and the fusion product assayed. The samples processed were taken from growing culture at 100 Klett reading. The β-galactosidase measurements revealed a distinct expression pattern among the four promoters relative to *rrnO,* the strongest promoter (Fig. 5). The *rrnJ* promoter also showed a high level of strong expression (73% of *rrnO*). The *rrnD* and *rrnB*

promoters on the other hand, showed much lower levels of β-galactosidase expression (18% and 6% of *rrnO,* respectively).

B- RNA Dot blot assay

The relative expression of the four *rrn* promoters and a *veg* gene promoter during growth in MM1 was determined at the RNA level by dot blot assay using a lacZ DNA probe. As shown in Fig 6 a distinct order of promoter strength was also observed, that is similar to what is observed using the β-galactosidase assay. In all the strains analyzed, *rrnO* is the strongest promoter, *rrnJ* is intermediate (~60% of the activity of *rrnO*), and *rrnB* and *rrnD* are the weakest (~20% of *rrnO*).

C-Primer extension analysis

The relative abundance of *rrn* promoter (P1, P2) transcripts observed in these five strains were visualized and quantitated by primer extension. The primer used was complementary to the 5' end of the *lacZ* gene. As shown in Fig. 7, two transcripts were observed for both *rrnO* and *rrnJ,* as expected. These promoters exhibited a high level of transcription from P2 and a low levels from P1. Only low levels of P2 transcripts were detected for the weak promoters *rrnB* and *rrnD.* The *veg* gene control showed one band as expected. This assay also demonstrated that *rrnO* is the strongest operon, *rrnJ* is intermediate (~60% the activity of *rrnO*) and *rrnB* and *rrnD* are both weak operons (~25% and ~12.5% of *rrnO* activity,

respectively) (Fig. 7). The *veg* gene which represents the control promoter has ~37% of *rrnO* activity. The band intensity measurements also showed that P2 transcripts for both *rrnO* and *rrnJ* are highly abundant relative to the P1 transcripts (in *rrnO* P2 is 6-7 fold higher than P1; and in *rrnJ*, a P2 is 4-5 fold higher than P1).

In summary, our initial studies carried by Widom with a *rrn-lacZ* fusion (pWR112) plasmid (1), a derivative of pDEB1(1) containing *E. coli* transelational signals, inserted into 7 out of 10 resident *rrn* promoters of *B. subtilis* revealed that they are not equivalent in their expression levels (1). These levels were correlated with their genomic location and were grouped into strong (*rrnO, rrnW*), intermediate (*rrnA, rrnJ, rrnE*) and weak (*rrnD, rrnB*) promoters (1). We now show that the heterogeneity in expression persists at the heterologous locus *amyE*, indicating that this heterogeneity in expression is not dependent upon genomic location. Although these four operons are originally located at map coordinate (minutes) 001, 010, 070, and 280, they continue to exhibit their intrinsic promoter strength at their new chromosomal position. The reproducible hierarchy of expression of the *rrn* promoters indicates that basic differences in intrinsic promoter strength among the ribosomal operons exist irrespective of their genomic location, growth conditions and the method of assay.

II. Control mechanisms of rRNA expression:

A- Stringent response

Ribosomal RNA synthesis is essentially switched off upon the accumulation of uncharged tRNAs induced by the addition of SH (which induces amino acid starvation) or αMG (which induces carbon energy source starvation) to growing *B. subtilis* cultures in synthetic medium (56). These Agents promote the accumulation of large amounts of (p)ppGpp and inhibit the synthesis of stable RNA in a *relA*$^{+}$ strain but not in the relaxed *relA*$^{-}$ strain (29, 31).

1. Accumulation of polyphosphoyrlated nucleotides

a. during nutritional stress as function of the *relA* system.

We confirmed the finding which showed that growing *B. subtilis* under amino acid starvation condition results in the accumulation of (p)ppGpp nucleotides that is dependent on the *relA* gene mechanism (31). Both the wild type *relA*$^{+}$ (IS58) and the relaxed *relA*$^{-}$ (IS56) strains were grown in low phosphate medium and treated with SH. In IS58, the levels of ppGpp and pppGpp increased within the first 10 min of exposure to the inhibitor SH. There was also a concomitant decrease of GTP (Fig 8, 9). In strain IS56, on the other hand, no accumulation of (p)ppGpp was observed at any time. We also showed a similar response under carbon source limitation conditions, where strains IS58 and IS56 were grown in low phosphate medium treated with αMG. The αMG treatment of

IS58 was accompanied by increased levels of ppGpp and pppGpp with a concomitant decrease of GTP within the first 10 min. Again, IS56 showed no response to αMG; no detectable amounts of (p)ppGpp and unchanged GTP levels (Fig 10, 11).

Similar studies were repeated using the spontaneous AT^R revertant L3 derived from strain IS56 (40). This strain differed from IS56 by its ability to respond to carbon source limitation (Fig. 10, 11), while still being unable to respond to amino acid starvation (Fig 8, 9). The levels of both ppGpp and pppGpp increased within the first 10 min of exposure to the inhibitor αMG. This was accompanied by a decrease in the GTP levels. This contrasts with SH treatment where no change in GTP levels were observed (Fig 8, 9).

The amino acid requirements of *B. subtilis* strains IS56 and L3 were established in a complex minimal medium (40). As shown in Table 3, the *relA* mutant revealed phenotypic requirements for histidine, phenylalanine and valine in addition to its auxotrophic requirements (*trp, lys*). The L3 revertant, on the other hand lost most of these partial amino acid requirements, except for the requirement for phenylalanine. It should be noted that these amino acid requirements were found in an enriched minimal medium containing all other amino acids, purines, pyrimidines, etc. and thus reflects a genuine phenotypic requirement (34, 40). The frequencies of sporulation and competency in the three *B. subtilis* strains (IS58, IS56, and L3) were compared, as shown in table 3. The

relA mutant is less competent and its ability to sporulate is slightly impaired, while L3 exhibits maximal levels of heat-resistant spores and continues to be competent.

b. during inhibition of RNA polymerase function.

It was reported earlier that the addition of rifampin (rif), an inhibitor of the initiation of ribonucleic acid synthesis, results in the accumulation of (p)ppGpp in both the wild type and relaxed strains of *B. subtilis* (48). We confirmed these observations in both strains IS58 and IS56. Cells were grown in low-phosphate medium and treated with (10 µl/ml) rifampin. Accumulation measurements showed increased levels of pppGpp and ppGpp with a concomitant decrease in the level of GTP in both IS58 and IS56 strains (Fig 12). To demonstrate the direct role of RNA polymerase in the rif response, a *rpoB18* mutation in the β subunit of RNAP which is rifampin resistant (rif^R), was introduced into the wild-type $relA^+$ and the $relA^-$ mutant by transformation crosses to produce the strains ISR58 and ISR56 (Table 2A). These two strains were examined for their ability to respond to either serine hydroxamate (Fig 13) or rifampin (Fig. 12). As expected, only ISR58 showed an accumulation of (p)ppGpp under amino acid starvation conditions. Neither the ISR58 nor the ISR56 showed any accumulation of (p)ppGpp after rifampin treatment (Fig 12). The rif^R mutant clearly is blocked in ppGpp synthesis. This indicates that the RNAP is involve in a second pathway for (p)ppGpp synthesis and has as yet an undefined biochemical role.

2- Differential response of *rrn* promoters and individual promoter elements from the *amyE* locus to stringent control:

To study the response of the individual promoter elements of *rrn* operons (*rrnO, rrnJ*) to stringent condition, and the response of tandem promoters (*rrnO, rrnJ, rrnD, rrnB*) the same stress conditions described in 1 were imposed. These studies were assayed in both *relA*$^+$ and *relA*$^-$ genetic background .

a- The response to SH treatment as assayed at the *lacZ* translational level.

The IS58 and IS56 strains (transformed with the different *rrn-lacZ* constructs) were grown in enriched minimal medium (MM1) and treated with SH. The β-galactosidase measurements of *rrnO* and *rrnJ* tandem promoters and individual promoter fragments in the wild-type *relA*$^+$ (IS58) showed that the tandem promoters of *rrnO* (O-P1P2) and of *rrnJ* (J-P1P2) had the highest levels of β-galactosidase activity. These levels dropped dramatically upon addition of SH. As shown in Figures 14 and 15. The P2 individual promoter of *rrnO* (O-P2) and of *rrnJ* (J-P2) have higher levels of β-galactosidase activity than the P1 individual promoter of *rrnO* (O-P1) and *rrnJ* (J-P1) respectively. The response to SH was more rapid for O-P2 and J-P2 when compared to O-P1 and J-P1. These results indicate that both promoters P1 and P2 of both *rrnO* and *rrnJ* operons are subject to the stringent response although P2 responds faster (Figures 14 and 15).

The level of β-galactosidase activity of *rrnO* and *rrnJ* promoter elements in the *relA* mutant (IS56) also showed that O-P1P2 and J-P1P2 were the highest and similar to the *relA*$^{+}$. O-P2 and J-P2 revealed higher levels of activity than O-P1 and J-P1 but neither promoter responded to SH. The β-galactosidase levels did not change over time following the addition of SH. In summary, the relative strengths of the three promoter fragments were similar to what was observed in the wild type *relA*$^{+}$ strain, except for the insensitivity to starvation conditions. In general, the β-galactosidase activity levels in the *relA*$^{-}$ background were lower than those observed in wild type cells (Table 4 and 5). This is perhaps due to the pleiotropic effects of the *relA* gene on the general metabolic activity of *B. subtilis* strains.

b- The response to SH as assayed at the lacZ RNA level:

Dot blot analysis was carried out in order to asses the levels of lacZ - mRNA present in these cells following SH treatment of both the stringent and the relaxed strains (Fig 16). In strain IS58, the *rrnO*-P1 and *rrnO*-P2 promoters were subject to stringent repression and responded with a 13-14% decrease in the level of lacZ mRNA (Fig. 16). As expected, strain IS56 was totally unresponsive and the lacZ mRNA levels from *lacZ* fusion to P1 or P2 promoter was unaltered (Fig. 16). The P2/P1 ratio in strain IS58 and IS56 before and after treatment with SH was 1.8, 2.3 and 3.8, 4.1, respectively indicating that even when the total population

of stable RNA transcripts is decreasing the overall abundance ratio (P1/P2) is maintained irrespective of genotype. With the intact *rrnJ* fragment, the solitary JP1, JP2 promoters were less active when compared to their *rrnO* counterparts (Fig. 7). These differences amounted to 44% in P1-P2, 53% in P1, and 54% in P2 (compare Figs. 16 and 17). Following SH treatment, the P2 *rrnJ* transcripts decreased to 19% while the *rrnJ*-P1 transcripts decreased to 41% (Fig. 17). Similar responses were observed using the β-galactosidase assay levels (Tables 4, 5). In the relaxed strain (IS56), the intact *rrnJ* and the solitary promoters were unaffected by inhibitor (see Fig. 18 and Table 5). Both the RNA dot blot and β-galactosidase assays gave comparable results (Table 5).

Our data indicate that heterogeneity exists among the different *rrn* operons in *B. subtilis* in the response to stringent conditions induced by SH. Dot blots assays were made on the five constructs *rrnO, rrnJ, rrnD, rrnB* and *veg* gene. The transcriptional activity of both *rrnO* and *rrnJ* decreased significantly (8-15 fold) at 30min and 60min following SH treatment (Fig. 18). Both *rrnB* and *rrnD* showed little response (1.3-fold to a 1.5-fold increase) while as, expected, the *veg* promoter control, continued to function (1.9-fold increase).

C- The response to Alpha Methyl Glucoside as assayed at the lacZ RNA level.

The P2 promoter responded to glucose starvation by a decline in lacZ-mRNA levels of 13 and 24% for *rrnO*-P2 and *rrnJ*-P2, respectively (Figures 19 and 20). The dual promoters (P1-P2) of *rrnO* and *rrnJ* in the *relA*$^{+}$ background exhibited a decrease in expression to levels of 29% and 20% respectively (Figures 19, 20). The solitary P1 promoters were totally insensitive to carbon deprivation, exhibiting only minor decreases in RNA levels to 86% for *rrnO*-P1 and 90% *rrnJ*-P1 (Figures 19 and 20). Despite the high levels of (p)ppGpp produced following the addition of αMG in the *relA*$^{+}$ and *relA*^{S} strains (Figures 10 and 11), lacZ mRNA level from P1 persisted. on the other hand RNA level from both P2 and the P1 -P2 tandem virtually disappeared (Figures 19 and 20). In contrast, in the *relA*$^{-}$ strain all promoter elements of *rrnO* and *rrnJ* either continued to function at sligtly higher levels (122-140%) or were unchanged (100-105%) (Figures 19 and 20). No (p)ppGpp accumulation occurred in the *relA*$^{-}$ strain.(Fig 10,11).

The effect of αMG on the synthesis of lacZ mRNA from the four ribosomal promoters *rrnO, rrnJ, rrnD, rrnB* and the *veg* gene was also studied (Fig. 21). The extent of the response to carbon-source limitation varied significantly. While *rrnO* and *rrnJ* decreased 7-8 fold. *rrnB* and *rrnD* revealed essentially no change or a weak response (1-2-fold decrease). The *veg* promoter was unaffected by SH treatment (Fig. 21).

In summary, the accumulation of (p)ppGpp under stringent conditions in the wild type *relA*$^+$ strain was accompanied by decreased levels of rRNA from *rrn* operons. The response of these operons, was clearly heterogeneous, where the promoters barely responded to stringent regulation while the strong promoters did.

d. The *in vivo* response of the *rrnO* promoters to stress conditions at its native location:

These studies were made through primer extension assays. Experiments were conducted using a primer unique for the *rrnO* operon sequence. Strains with three genetic background *relA*$^+$, *relA*$^-$, and *relA*^{S} were used in these studies.

1- The response to SH treatments

The three cell lines IS58, IS56, and L3 were grown in MM1 medium and treated with SH for 60min to induce amino acid starvation conditions. RNA was isolated from samples taken at 0 and 60min following SH addition and used for primer extension analysis. Two transcripts were resolved, a low molecular weight band (39 bp) representing P2 and a high molecular weight band (150 bp) representing a transcriptional start site of P1 (Figures 22 and 23). In all three cell lines, the transcript ratio P2/P1 prior to treatment was between 2.3 and 8.1 confirming that P2 is the active promoter. In *B. subtilis* the band intensities for both transcripts decreased 60min after treatment. The P2 transcript exhibited a

stronger response to SH than did P1. P2 showed an 85% decrease in activity, while P1 activity only decreased 30%. This study also demonstrated that both P1 and P2 is inhibited by SH in IS58. P1 and P2 promoters in both IS56 and L3 are insensative to SH, (the transcriptional activity if any thing continue to increase slightly 60 min. after SH) (Fig 23).

2- The response to αMG treatments

The RNA was isolated from cultures at 0 and 90min after inducing carbon source limitation by αMG treatment (Fig. 24). The transcription ratio of P2/P1 in all three strains was also high and ranged between 4.7-10. This is similar to what was observed the SH studies. The P2 promoter from both strains IS58 and L3 displayed an 80- 85% decrease in activity following 90min of treatment with αMG. On the other hand, P1 transcripts increased by 1.6 fold in IS58 and 4.4 fold in L3. In the relaxed strain, IS56, neither P1 nor P2 transcript exhibited a reduction. (Fig. 24). During carbon source limitation, the primer extension assay clearly revealed that P2 of *rrnO* is differentially regulated with respect to P1 in *relA*$^+$ and the *relA*^{S} strains but not in the *relA*$^-$ background.

We conclude that the tandem ribosomal promoters P1 and P2 are regulated differentially and depending on the treatment used, the P1 promoter either exhibits a relaxed response to carbon source transitions or a stringent response to amino acid deprivation.

3. Rifampin treatments

The relative abundance ratio of P2/P1 transcripts was 5.1 for IS58 and 5.5 for IS56. As shown in Figure 25, within the first 5 min of rifampin treatment (10 μg/ml), the levels of P2 transcript decreased to 2.8% in *relA*$^+$ cells and 5.1% in *relA*$^-$ cells. At the same time, levels of P1 transcripts declined to 32% in *relA*$^+$ cells and 35% in the *relA*$^-$cells. The P2 promoter of *rrnO* responds swiftly to transcriptional inhibition induced by the inhibitor of the β -subunit of RNAP. This effect is similar to what was observed following both SH and αMG treatment. It show more decay of P2 mRNA than P1 mRNA.

B- Growth rate control

1- Growth rate response of *rrn* promoters:

The effect of different growth media on *rrn* operon expression was examined in *B. subtilis*. These measurement were made on the heterologous locus (*amyE* gene) (Figures 26, 27). The β-galactosidase activities of the four *rrn* promoters (*rrnO, rrnJ, rrnB, rrnD*) were determined from cultures grown in different media, MM1, MM2, and MM3. The doubling times per hour were 0.60, 0.35, and 0.25, respectively. All four *rrn* operons demonstrated lower levels of β-galactosidase activity when grown in MM2 and MM3 media as compared to what is observed in cells grown in MM1 medium. The activities of *rrnO* decreased to 75% in MM2 and 62% in MM3, *rrnJ* decreased to 45% in MM2 and 9% in MM3,

rrnB decreased to 53% in MM2 and 33% in MM3 and *rrnD* decreased to 15% in MM2 and 50% in MM3. The data shows that the four *rrn* operons used in this assay displayed growth rate response.

To investigate the response of the individual promoter to growth rate control, similar measurements of the *rrnJ-P1 and rrnJ-P2* expression in *lacZ* fusion were made. The two strains containing the appropriate fusion were grown in the four growth media VY, MM1, MM2, and MM3 (Fig. 28). These data reveal that, both promoters P1 and P2 are growth rate regulated. They both exhibit high levels of expression in the rich media (VY, MM1) and low levels of expression in the poor media (MM2, MM3). However, these data also indicate that P1 promoter shows less growth regulation than does P2 (Fig. 28). Since the two promoters are growth regulated in both the wild type and the *relA* mutant, (p)ppGpp is not likely to have a major role in growth rate control.

2- The role of (p)ppGpp in growth rate regulation:

We hypothesized that if (p)ppGpp has a role in the growth rate control, there must be another pathway responsible for the expression of the (p)ppGpp. Furthermore, we believe that it is through this second pathway that cells maintain their growth rate control. Based on previous studies (48) and our studies discussed above, we postulate a role for RNAP in such a pathway. Since our studies with rifampin suggested that RNAP is involved in a second pathway of

(p)ppGpp synthesis, we decided to use a *B. subtilis* strain that has a mutation in the RNAP β-subunit and a mutation in the *relA* gene in order to ensure the inactivation of (p)ppGpp synthesis from both pathways. The three cell lines strains ISR58, ISR56, and LR3, all containing the two *lacZ* fusions *rrnO* and *veg* gene were grown in the three media (MM1, MM2, and MM3) and the levels of the fusion product determined.

The β-galactosidase activity studies of *rrnO* showed that the expression was growth rate regulated in the ISR58 strain where there was high β-galactosidase activity levels in MM1 medium and low levels in both MM2 and MM3 (35% and 40% of the activity observed in MM1 respectively) (Fig. 29, 30). The same was found for LR3 strain where the activity relative to what was observed for MM1 decreased to 35% in MM2 and 40% in MM3. On the other hand, ISR56 showed similar levels of β-galactosidase activity in all three media, suggesting that this cell is unable to respond to different media.

The RNA dot blot studies corroborate these findings. A high level of *rrnO-lacZ* fusion RNA is detected in ISR58 grown in MM1 medium. In MM2 the level falls to a bout 25% of what is observed in MM1 and even lower in MM3 abut 12% of MM1 (Fig. 31, 32). The *rrnO-LacZ* transcription activity in the L3R3 strain was also less in MM2 and MM3 when compared to MM1(40% less than MM1 for both MM2 and MM3). The relative amounts of mRNA-lacZ in ISR56 showed smaller differences between the different media when compared to those observed in both

the ISR58 and LR3 strains. The *rrnO* activity in the ISR56 strain was only 83% of MM1 activity when grown in MM2 and 50% of the MM1 activity when grown in MM3 medium.

These findings show that if only the *relA* gene is mutated, the *B. subtilis* strain will remain growth rate regulated because there is still *relA*-independent RNAP-dependent (p)ppGpp synthesis taking place. However, in the (p)ppGpp deficient strain which has a double mutation (*relA, ropB18*), growth rate regulation is affected (less growth rate regulation).

Concluding Remarks

Although there is little variation in the primary sequence of ten ribosomal RNA genes in *B. subtilis* and the seven in *E. coli,* they respond differently to a variety of physiological conditions. In the present study, we examined the activity of four specific rRNA promoters as single-copy integrants into the *amyE* locus and these activities were compared to earlier studies were 7/10 *rrn* genes integrated at their native loci via single-copy insertional plasmid (1). Studies on *rrn* expression in both complex and different minimal media have shown that ribosomal promoters differ significantly from one another in strength, the strongest being *rrnO* and *rrnJ* and the weakest *rrnD* and *rrnB*. In both *B. subtilis* and *E. coli* the level of β-galactosidase or CAT activity observed in fusions grown in various

media correlated well with their genomic location relative to the oriC (1, 67), when expression is measured from the native locus.

The heterogeneity in the pattern of expression seen with the four *rrn* operons used in this study is still observed even though they were all measured from the same chromosomal location (*amyE* locus). It appears on the basis of these data, that only the immediate UAS region is critical and not the larger distance from oriC region. The finding that the *rrn* operons activities appears to correlate with their original location could be coincidental.

The sequence heterogeneities seen among *B. subtilis* rRNA gene sets could be responsible for differential rates of rRNA processing and may also affect message stability, thus modulating the contribution of individual gene sets post-transcriptionally. Certainly the 65bp. insert found in the leader region of *rrnO, rrnB,* and *rrnD* has no affect on the relative promoter strength because it exists in both strong and weak promoters. The two largest tRNA gene clusters, *trnD* (16-tRNA genes) and *trnB* (21-tRNA genes) are symmetrically situated downstream from the two weakest operons (*rrnD* and *rrnB*) and are farthest away from the oriC region (8). Since these tRNA genes are actually part of the rRNA transcriptional unit (6), it has been suggested that this could offer a reason for the differences in strength or temporal regulation for the 10 ribosomal operons in the endospore forming *B. subtilis* (69). To date, no correlation seems to exist between the strength of

ribosomal promoter and the presence or absence of downstream tRNA genes (69, 70).

The effect of the amino acid analogs such as SH and nonmetabolizable carbohydrates like αMG on the expression of the tandem promoters of *rrnO, rrnJ, rrn*B and *rrnD* was studied. The two operons *rrnO* and *rrnJ* displayed a strong response to both analogs while *rrnB* and *rrnD* showed almost no response to either. This observation reveals the presence of heterogeneity in the regulation among the four *rrn* operons used in this study. It is clear that the rRNA operons in *B. subtilis*, like the seven rRNA operons in *E. coli*, are neither expressed nor regulated equally. Rather that expression of individual rRNA operon can be affected differently by both physiological conditions and their unique upstream sequence .

The *B. subtilis* strains tested respond to amino acid starvation and carbon source limitation by an increase in (p)ppGpp levels via a *relA* gene-dependant mechanism. In addition to the nucleotide accumulation, the rRNA synthesis is subjected to stringent control by showing a drastic reduction in rRNA levels. In this study, we demonstrated the inverse relationship between the accumulation of (p)ppGpp and the decrease in rRNA levels. The *relA*$^{+}$ strain (IS58) showed under conditions of amino acid starvation or carbon source limitation, there is both an accumulation of (p)ppGpp, and a reduction in rRNA levels. The *relA*$^{-}$ strain (IS56) neither showed accumulation of (p)ppGpp nor a reduction in rRNA synthesis, in

either of the two stringent conditions. The *relA*$^{(S)}$ L3 behaved like the IS56 in response to amino acid starvation conditions, but accumulated (p)ppGpp and showed a reduction in rRNA levels under the carbon source limitation conditions. The L3 revertant (*relA/relA*$^{(S)}$) represent an intragenic suppression event that yielded partial restoration of the wild-type phenotype specifically, the AT^{R} locus, the partial loss of amino acid requirement; and the ability to respond to carbon limitation. The *relA*^{s} behaves like a suppressor mutant and phenotypically resembles the original *relA* mutant of *E. coli* (40). On the other hand, the original *relA*$^{-}$ mutant strain IS56 seems to resemble the Δ*relA*, Δ*SpoT* mutant of *E. coli*, in which requirement for a whole set of amino acid was found (34). These results suggest that the *relA* locus in *B. subtilis* appears to be subdivided into two domains. The first domain is involved in the response to amino acid limitation, while the second domain is the putative *relA*^{S} allele involved in the response to carbon source limitation. Thus, the original mutant isolated in *B. subtilis* (31) can be regarded as a double mutant with defects in both activities.

Accumulation of (p)ppGpp was also observed in both wild-type RNAP strains IS58 and IS56 following treatment with rifampin. Rifampin binds to the β-subunit of RNAP, thus blocking the initiation of transcription. It is thought that the RNAP is involve in (p)ppGpp metabolism while in the free or initiating state (48). Although rifampin has been used to block initiation of transcription, other evidence has shown that it only partially antagonizes dinucleotide synthesis and σ subunit

release (52). Our results confirm the notion that there is a second mechanism responsible for (p)ppGpp accumulation, a reaction which is dependent on free or initiating RNA polymerase molecules and independent of the normal ribosome-mediated process (48).

Previous experiments in *E. coli* and sequence comparison of all known stringent regulated promoters reveals the presence of a highly conserved GC-rich discriminator sequence located between the -10 region and the transcription start site. For the *E. coli* ribosomal RNA promoter, the consensus discriminator sequence GCGC is found only in P1 promoters and not in P2 promoters (72). A single base substitution (A to G at position -6), changed the response of the *E. coli* *rrnB* promoter P2 to that of a stringent control as well as growth-rate regulation (72). In at least the seven sequenced *rrn* promoter regions of *B. subtilis* (1, 8, 12, 23, 33), a discriminator motif sequence is found in both promoters P1 and P2. The consensus sequence [GT(C/T)G(C/T)T(T/Pu)] is structurally more degenerate and varied than what is observed in *E. coli*. In addition, in *B. subtilis* it is located further downstream at the initiation site of transcription. To date, no attempts to mutate the *B. subtilis* target sequence have been reported. Our laboratory has recently initiated *in vitro* mutagenesis of O-P1 in order to ascertain whether or not mutations in this region produce a promoter that responds to carbon starvation.

We have examined the effect of both SH and αMG on the expression of the individual promoter elements of *rrnO* and *rrnJ* fused to a *lacZ* reporter gene.

Unlike the normal situation in *E. coli*, both promoters (P1 and P2) were affected by SH. The downstream P2 promoter did however, respond immediately and to greater extent than did the P1 promoter. We found that upon carbon starvation, little or no activity of the downstream promoter (P2) was detectable, while P1 activity persisted. This finding is similar to what is found in *E. coli* where the dual promoters are differentially regulated but in an opposite manner: namely, under both nutritional stress conditions, only P1 responds while the downstream P2 is not stringently controlled (1, 24, 71). In *E. coli,* in the absence of P1, the P2 promoter becomes active and is subject to stringent control. The activity of solitary P2-*rrnA* decreased following the addition of SH in a wild type strain but not in a Δ*relA* strain (47). The authors concluded that the *E. coli rrn*-P2 promoters are both efficient and highly regulated. However, they found that in the normal tandem arrangement (P1-P2) the P2 promoter is inhibited by transcription initiated at the upstream P1 promoter (47). Similarly, we found by primer extension analysis of the natural tandem promoter configuration of *rrnO* in *B. subtilis*, that P2/P1 ratios of transcriptional activities were 3-4 fold higher than those obtained for the solitary promoters. In *B. subtilis*, we see an apparent inhibition of the upstream P1 promoter during transcription from the active P2 promoter. This inhibition was in part alleviated in the solitary construct. An *in vitro* study on the effect of ppGpp on the kinetics of formation of transcription complexes between *E. coli* RNA polymerase and the *rrnB*-P1 and P2 in *B. subtilis* has shown that ppGpp does not

differentially inhibit B-P2 at any stage (68). Taken together, these results indicate that individually the promoters are efficient and are likely to be similarly regulated.

In contrast to *E. coli* promoters, the *B. subtilis* P2 promoters are transcriptionally the more active of the P1- P2 pair. However both P1 of *rrnO* and *rrnJ* are growth rate- regulated and stringently respond to amino acid starvation. The main expression heterogeneity lies in their differential response to carbon-source limitation. Although transcription from *B. subtilis rrnB*-P2 and *rrnJ*-P2 is subjected to growth rate-dependent control in *E. coli*, they have very little sequence similarity to the *E. coli* growth rate-regulated *rrnB*-P1 (1, 24, 68) This lack of homology is difficult to reconcile, since all evidence suggests that it is the core promoter region which acts as the target for regulating the growth rate-dependent expression of an *rrn* promoter (1, 13). Constructing promoter fragments of *rrnO*- P1-P2 without the UAS for in vitro transcription assay using either the *B. subtilis* or the *E. coli* RNA polymerase have yielded a strong bias in the amount of the P2 transcripts produced relative to P1 (unpublished results of D. Liu). Although these fragments were devoid of the UAS, which is known to activate transcription more than 300-fold in *E. coli* (48) and only 10- fold in *B. subtilis,* they still contained the UP element, a *cis* acting DNA sequence between -60 and -40 that may be responsible for the differential promoter strengths (Table 2B). There are subtle sequence heterogeneities in the UP elements of the individual promoter elements of *rrnO* and *rrnJ,* where the AT content is clearly higher in P2 when

compared to P1 namely, 20 AT: 4 GC versus 13- 17 AT: 11-7 GC, respectively (Table 2B). Similarly there are minor sequence variations between P1 and P2 in the discriminator region of the 7/10 sequenced *B. subtilis rrn* promoters. All the P2 discriminators end with TT while most of the P1 discriminator begin with GTT including the single P1 promoters from tandem sets of operons (*rrnJ- rrnW* and *rrnI-rrnH-rrnG*; 1, 8, 12, 33). In summary, although there are some obvious examples of sequence heterogeneities upstream and downstream of the promoter elements P1 and P2, their final role in intrinsic transcription strength, growth rate-control and differential stringent response will have to await a systematic modification and deletions of key nucleotides

Reduction of rRNA synthesis from the *rrnO* operon was observed after rifampin treatment. This reduction in rRNA synthesis took place in both strains IS58 and IS56. Both promoters P1 and P2 displayed a reduction in activity, but the P2 promoter showed a stronger response than did P1. We demonstrated that when *B. subtilis* cells adapt to the new nutritional stress conditions which lead to blocking the initiation of transcription, there is a second pathway responsible for (p)ppGpp accumulation. This pathway is different from the *relA* pathway and appears to act through the direct involvement of RNAP. This accumulation of phosphorylated compounds is also accompanied by a reduction in rRNA levels confirming the notion that (p)ppGpp is one of the major regulatory signals during

stringent response and that its targets occur both at the metabolic level and at the level of RNA transcription initiation (48).

Our early studies with a *rrn-lacZ* fusion for *rrnO, rrnA, rrnJ, rrnB, rrnW, rrnE* and *rrnD,* using plasmids integrated at their homologous chromosomal positions, revealed that they responded to growth rate regulation with a 2-4 fold increase in the levels of β-galactosidase activity after a shift-up from minimal medium (MM1) to complex medium (VY) (1). The β-galactosidase activities from the *rrn- lacZ* fusions used in this study (*rrnO, rrnJ, rrnD, rrnB*) were also measured in different media (MM1, MM2, and MM3) but these measurements were conducted from the heterologous locus (*amyE*). These constructs were shown to respond to growth rate regulation. we conclude that growth rate regulation of *rrn* operons in *B. subtilis* is largely unaffected by the chromosomal location relative to the origin of replication. we base this conclusion on the fact that all *rrn* operons used in this study were measured from the same heterologous locus and showed a pattern of growth rate regulation similar to what is found when they were measured from the original location (1). This finding contrasts with a study done in *E. coli* which indicates that the clustring of *rrn* operons close to the origin of replication is likely to account for the growth rate dependent of *rrn* synthesis (4).

SUMMARY

1. A single copy integration plasmid pDH32/pDG268 was used to create *rrn-lacZ* fusions in the chromosome of *B. subtilis.*

2. The heterologous measurements of the β-galactosidase levels and the lacZ mRNA levels of the *rrn-lacZ* fusion for the four RNA operons, show three levels of activity; strong (*rrnO*), intermediate (*rrnJ*), and weak (*rrnD, rrnB*).

3. The strong and the intermediate operons (*rrnO, rrnJ*) are located close to the origin of replication, and the weak operons (*rrnB, rrnD*) are located a way from the origin of replication.

4. In the four *rrn* operons used in this study (*rrnO, rrnJ, rrnD, rrnB*), the P2 promoter is the strong promoter and P1 is the weak promoter.

5. The wild type *relA*$^{+}$ strain accumulates (p)ppGpp under amino acid starvation and carbon source limitation conditions, while the *relA*$^{-}$ mutant does not.

6. It is suggested in this study that there is a second mechanism responsible for (p)ppGpp accumulation, a reaction which is dependant on free or initiating RNA polymerase molecules and independent of the normal ribosome-mediated process.

7. Both promoters (P1, P2) in the *rrnO* and *rrnJ* operons respond to amino acid starvation conditions by showing a decreas in activity but the downstream

promoter (P2) revealed a stronger and a faster response than the upstream promoter (P1).

8. Only the downstream promoter (P2) in *rrnO* and *rrnJ* responds to carbon source limitation condition, where the rRNA synthesis diminishes. The upstream promoter (P1) does not respond.

9. The *rrnO* and *rrnJ* operons in the *relA*^{S} strain show response to carbon source limitation conditions similar to the response in the *relA*$^+$ strain; but under amino acid starvation they exhibited relaxed rRNA synthesis as the *relA*$^-$ mutant strain .

10. The four *rrn* operons (*rrnO, rrnJ, rrnB, rrnD*) studied from the heterologous region (*amyE* locus) are growth rate regulated.

11. Both promoters of *rrnJ* operons were growth regulated.

12. The tandem promoter of *rrnO* in *a B. subtilis* strain with the double mutations *relA, rpoB18,* (both genes involved in (p)ppGpp synthesis pathway), demonstrated a lack of growth rate control. This indicate that (p)ppGpp has role in growth rate control.

TABLE 1

PROMOTER REGIONS OF *B. subtilis* AND *E. coli rrn* OPERONS

	P1				P2				m16S
	-35 bp.[a]		-10 bp.		-35 bp.		-10 bp.		
***B. subtilis*[b]**									
rrnO	TTTACA	16	TATAAT	69	TTGACC	17	TACTAT	179	TTTAT
rrnA	TTGACT	17	TATTCT	50	TTGACA	17	TATGAT	117	TTTAT
rrnJ	TTGCAC	17	TATATT	57	TTGACT	17	TATAAT	118	TTTAT
rrnH	--		-- --	-	TTGACT	17	TATACT	122	TTTAT
rrnG	--	--	--	-	TTGACT	17	TATACT	123	TTTAT
rrnD	TTGACT	17	TATATT	68	TTGACA	17	TATATT	182	TTTAT
rrnB	TTGCAA	17	TATATT	63	TTGACA	17	TATATT	178	TTTAT
***E. coli*[d]**									
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) Bases in between the consensus sequences were counted starting with the first nucleotide after the consensus box and ending with the nucleotide preceeding the next consensus box or m16S 5' terminus.

(b) References for *B. subtilis* sequences are *rrnO*, *rrnA* (12), *rrnJ* (1), *rrnH* (6), *rrnG* (6), *rrnD* (23), *rrnB* (33).

(d) *E. coli* were taken from (4).

TABLE 2A. *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
L3	*trpC2 lys-3 relA*$^{(S)}$		R. Rudner(40)
1A308	W168, *rpoB18*		BGSC[a]
1A500	*rpoB1*		BGSC[a]
LS11	*tryE26 leuA1 rpoB500*		BGSC[a]
ISR58	*trpC2 lys-3 relA*$^{+}$ *rpoB18*		W. Samarrai
ISR56	*trpC2 lys-3 relA*$^{-}$ *rpoB18*		W. Samarrai
LR3	*trpC2 lys-3 relA*$^{(S)}$ *rpoB18*		W. Samarrai
II.PLASMIDS[b]			
pDH32	*amp*^{R},*cm*^{R},*amyE*,*spoVG-lacZ*	9.9	D.Henner(58)
pDG268	*amp*^{R},*cm*^{R},*amyE*,*spoVG-lacZ*	9.3	P.Stragier(57)
pPW4[c]	pDH32-(P1-P2)-*rrnO*	1.9Kb	C. Stewart
pAWR118	pDG268-(P1-P2)-*rrnJ*	1.5 Kb	A. M. white
pAWR116	pDG268-(P1-P2)-*rrnD*	2.2 Kb	A. M. white
pAWR123	pDG268-(P1-P2)-*rrnB*	1.8 Kb	A. M. white
pPW810[c]	pDH32-(P1)-*veg*	0.48 Kb	C. Stewart
pLR105	pDG268-(P2)-*rrnJ*-269bp		D. Liu
pLR210	pDG268-(P1)-*rrnJ*-194bp		D. Liu
pLR203	pDG268-(P1-P2)-*rrnJ*-441b		D. Liu
pLR201	pDG268-(P1-*Hind*III-P2)-*rrnJ*-463bp		D. Liu
pLR501	pDG268-(P2)-*rrnO*-341bp		D. Liu
pLR512	pDG268-(P1)-*rrnO*-240bp		D. Liu

(a) Bacillus Genetic Stock Center, Columbus, Ohio
(b) All plasmids listed were introduced into strains IS58, IS56 and L3.
(c) Plamids introduced to strains ISR58 and ISR56.

TABLE 2B. The sequences of *B. subtilis rrnO* and *rrnJ* promoter elements used in this study

Promoter	Sequence[a]	Regulation[b] SHX	Regulation[b] αMG
rrnO-P1	CTGCGCTTTTTTGTGTCATAACCCTTTACA GTCATAAAAATTATGGTATAATCATTTCTGTTGTCT	+	-
rrnO-P2	AGTAAAAACTTTTTCAAAAAAGTATTGACCTAGTTAACTAAAAATGTTACTATTAAGTA GTCGCTT	+	+
rrnJ-P1	CCAGTAGTATTTCTTCAAAAAAACTTGCACTATTATTTACTAGGTGGTATATTATTATTCGTTGCCG	+	-
rrnJ-P2	AAAAAAGAACTTCAAAAAAAGTTATTGACTTCACTGAGTCAAGGAGTTATAATAATAAAGACGTACC	+	+
	UP element -35 -10 Discriminator		

[a] The *B. subtilis* promoter sequuences of *rrnO* and *rrnJ* are from Ogasawara et. al. 1983 (12) and Widom 1988 (1) respectively.
[b] The ability (+) or inability (-) of a promoter to be regulated under nutritional stress conditions SHX or αMG is indicated.

TABLE 3

THE MUTANT STRAINS OF *B. subtilis* USED IN THIS STUDY

Relevant genotype[a]	Strain[b]	Phenotypic requirement of aa[c]	Resistance[d] to:		Accumulation of (p)ppGpp[e] in:		Sporulation frequency[f] (%)	Transformation frequency[g] (%)
		MM-X	AT	Ts	SH	αMG	NSM	
Wild type (*relA*⁺, *relC*⁺)	IS58	(trp,lys)	R	S	+ +	+ +	76	100
relA	IS56	(trp,lys) phe,his,val	S	S	-	-	48	9
relA, *relA*[e]	L3	(trp,lys) phe	R	S	-	+	85	46

[c]MM-X = phenotypic aa requirement noted as 20aa-X in minimal medium containing an incomplete set of 19 aa with the missing X indicated, the aa in parethesis represent the auxotrophic requirement of the strain.
[d]AT = 3-amino- 1,2,4 triazole (15-30 mM); Ts = thiostrepton (1-5 μg/ml)
[e]SH = Serine hydroxamate (1-2 mg); αmG = α-methyl glucoside (1%).
[b]Strains IS58, IS56 obtained from I.Smith.

Table 4

THE EFFECT OF SERINE HYDROXYMATE ON THE EXPRESSION OF THE *rrnJ* PROMOTER ELEMENTS AS ASSAYED BY β-GALACTOSIDASE ACTIVITY AND RNA DOT BLOTS

		IS58 relA⁺ (*trpC2, lys-3*)				IS56 relA⁻ (*trpC2, lys-3*)			
Promoter	SH* (Min.)	Miller Units	Percent	RNA**	Percent	Miller Units	Percent	RNA**	Percent
J-P1, P2	0	1,420	100	16,950	100	590	100	12,650	100
	30		60	2,197	13		140	17,233	136
	60		31	1,155	7		122	21,809	172
J-P1	0	500	100	6,627	100	450	100	5,357	100
	30		51	2,614	39		121	8,049	150
	60		13	5,070	76		114	6,679	125
J-P2	0	1,040	100	10,743	100	400	100	10,591	100
	30		40	1,869	17		161	16,466	155
	60		13	1,994	19		116	13,908	131

*SH: serine hydroxamate 2 mg/ml in MM1
[Spizizen salts plus 0.5% glucose - 1% Na glutamate]
**RNA: relative hybridization to a *lacZ* probe

Table 5

THE EFFECT OF SERINE HYDROXYMATE ON THE EXPRESSION OF THE *rrnO* PROMOTER ELEMENTS AS ASSAYED BY β-GALACTOSIDASE ACTIVITY AND RNA DOT BLOTS

		IS58 relA⁺ *(trpC2, lys-3)*				IS56 relA⁻ *(trpC2, lys-3)*			
Promoter	SH* (Min.)	Miller Units	Percent	RNA**	Percent	Miller Units	Percent	RNA**	Percent
O-P1, P2	0	995	100	42,215	100	575	100	10,408	100
	30		35	4,120	10		127	15,843	155
	60		27	5,031	12		118	14,012	135
O-P1	0	460	100	13,144	100	285	100	2,676	100
	30		78	4,279	33		95	2,914	108
	60		27	--	—		98	2,504	93
O-P2	0	595	100	21,956	100	380	100	7,901	100
	30		30	2,603	12		109	10,290	130
	60		29	2,021	9		84	11,204	142

*SH: serine hydroxamate 2 mg/ml in MM1
[Spizizen salts plus 0.5% glucose - 1% Na glutamate]
**RNA: relative hybridization to a *lacZ* probe

Figure 1. 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 reported by Rudner et. al. 1993 (6). The number of tRNA in each cluster is indicated in the parenthesis. The map is based on the genetic mapping reported by Jarvis et. at. al. 1988 (7). The basic reference for the known loci is taken from the mapping kit strains of Dedonder et. at. al 1977 (10).

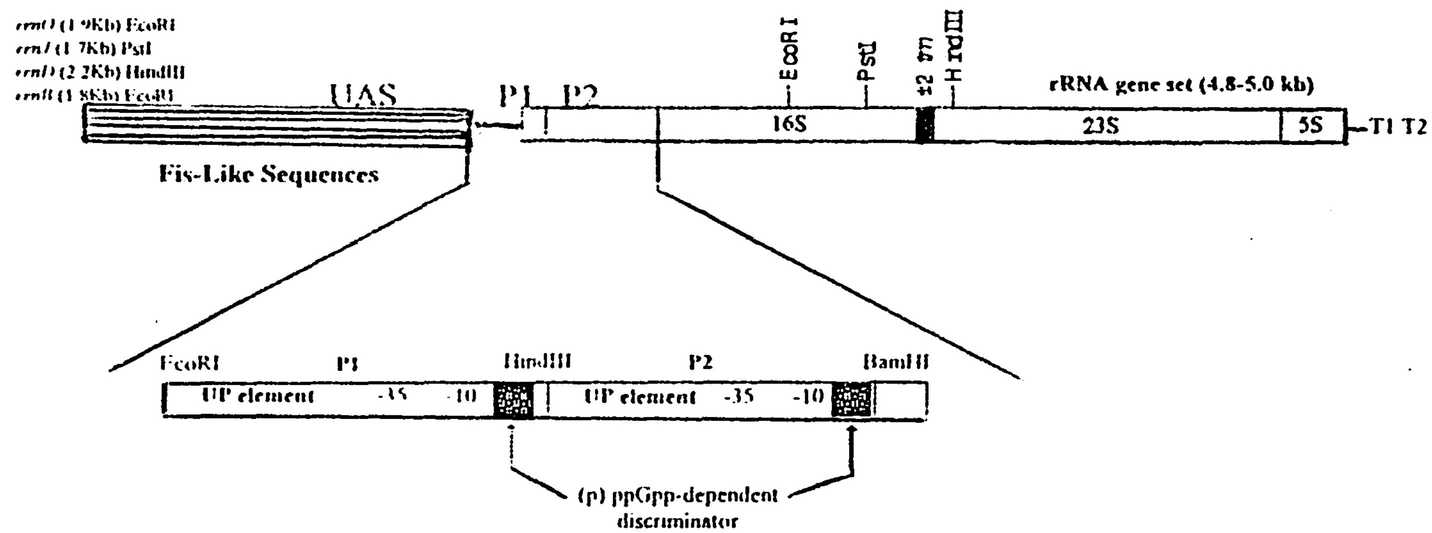
168

360/0
90
180
270

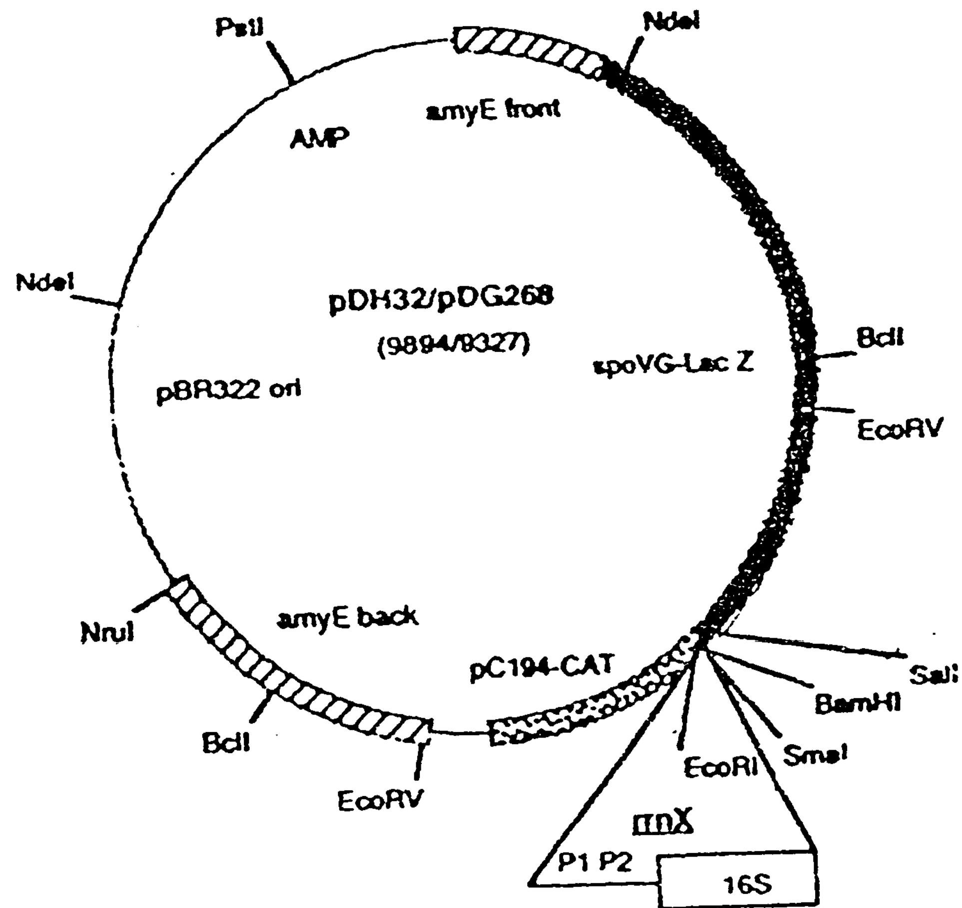
O
rrnO-trnO(2)
rrnA-trnA(2)
rrnJ-trnJ(9)
rrnW
rrnI-trnI(6)
rrnH
rrnG
trnS(7)
rrnE-trnE(2)
rrnD-trnD(16)
rrnB-trnB(21)
cysA
att(SPO2)
amyE
aroI
dal
pheA
purB
tre
thiA
glyB
metD
metC
rpsP(S16)
pyr
hisA
thyA
Ts
ilvA
trpE
aroD
ilvC
leu
rplU(L21)
rpmA(L27)
aroG
ald
thrB
cysB
hisA
ctrA
sacA
rpsF
rpl(L17-34)
rps(S3-20)

Figure 2. Generalized map of *rrnO* tandem promoters and promoter fragments. PCR amplifications of individual promoter elements were created using pGEM-*rrnO* and pGEM-*rrnJ* plasmids as templates. The P1 fragments are flanked by two restriction sites *EcoR*I and *HindIII* and the P2 fragments by *HindIII* and *BamHI* and were cloned in pDG268 by D. Liu (see Table 2A, B).

Generalized Map of *Bacillus subtilis rrn* Operons & Promoter Regions



Figures 3. Single-copy integration vectors (pDH32/pDG268) for *B. subtilis* that allow the targeted integration of a single copy of a *lacZ* fusion into the *amyE* gene. The essential features of these plasmids are that they contain a promoterless *lacZ- spoVG* with a *B. subtilis* RBS (Ribosomal Binding site= translational signals) and a CM^R determinant, flanked by larger regions of *amyE*. In pDG268, an *EcoRI-HindIII- BamHI* polylinker has been introduced. Four *rrnX* promoter fragments (*rrnO, rrnJ, rrnB, rrnD*) and the individual promoter fragments of *rrnO,* and *rrnJ* were introduced into these plasmid by transformation of the *PstI* linearized plasmid and selection on CM plates.



rrnX

a. pPW4-*rrnO*/ pDH32

b. pAWR116-*rrnD*/ pDG268

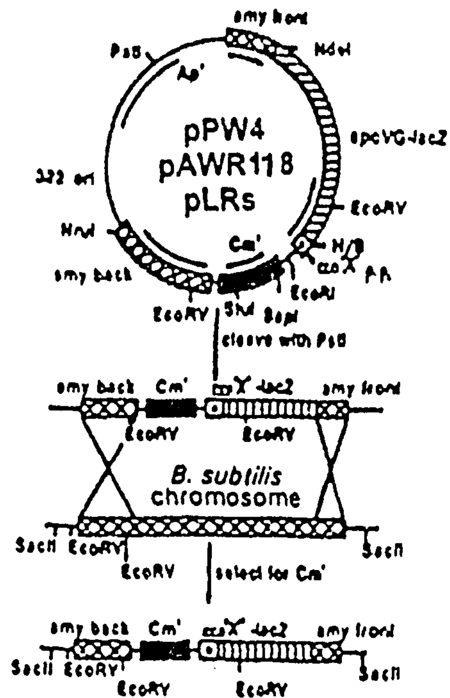
c. pAWR123-*rrnB*/ pDG268

d. pAWR118-*rrnJ*/ pDG268

e. pPW810-*veg*/ pDH32

Fig. 4. Illustration of the integration of pDH32/pDG268 and its derivatives at the *amyE* locus of *B. subtilis*. a- Shows the replacement integration by double cross over replacing the middle part of the *amyE* gene on the chromosome with the new construct yielding an *amyE*$^-$ strain. b- Shows the insertion integration were the plasmid integrate in the chromosome by a Campbell type insertion yielding an *amyE*$^+$ strain.

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



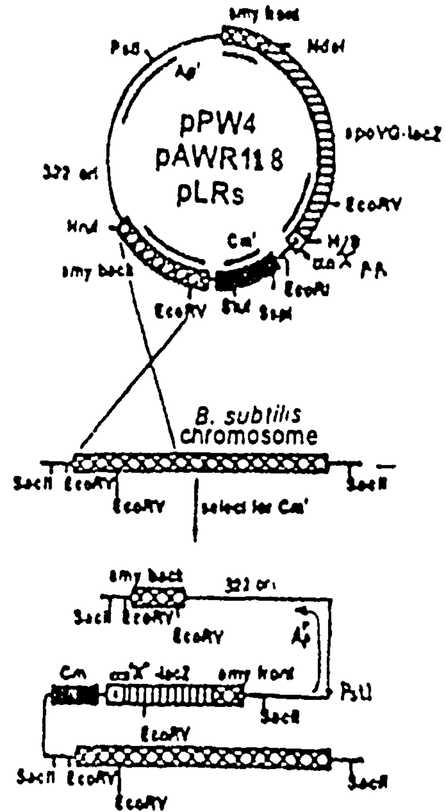


Fig. 5 Relative promoter strength of four *rrn* operons as measured by β-galactosidase assay by the method of Miller (61). β-galactosidase produced by *B. subtilis* strain IS58 containing integrated lacZ fusion: pPW4-*rrnO*, pWAR118-*rrnJ*, pAWR123-*rrnB*, and pAWR116-*rrnD* . One milliliter cell samples from cultures growing in MM1 medium were taken at a reading of 100 Klett units.

The relative promoter strength of the four rRNA operons as assayed by B- galactosidase

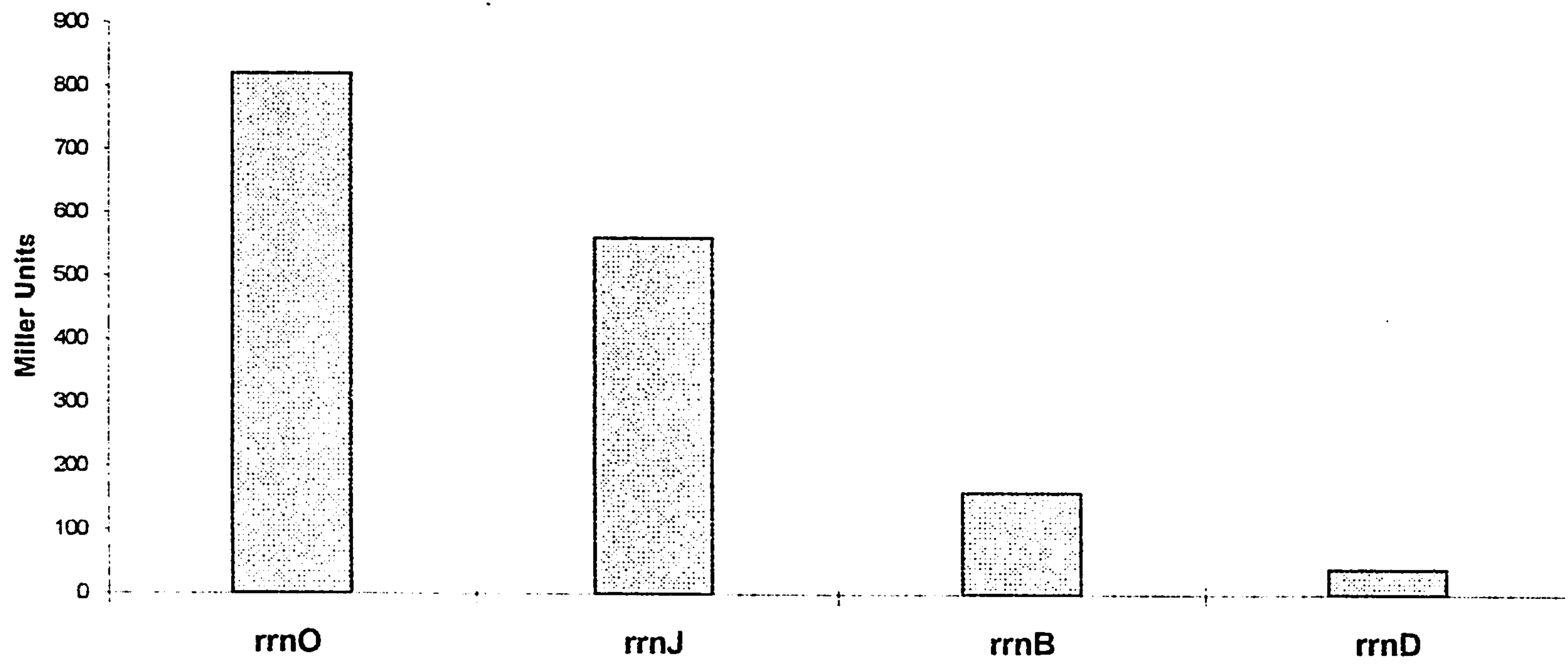
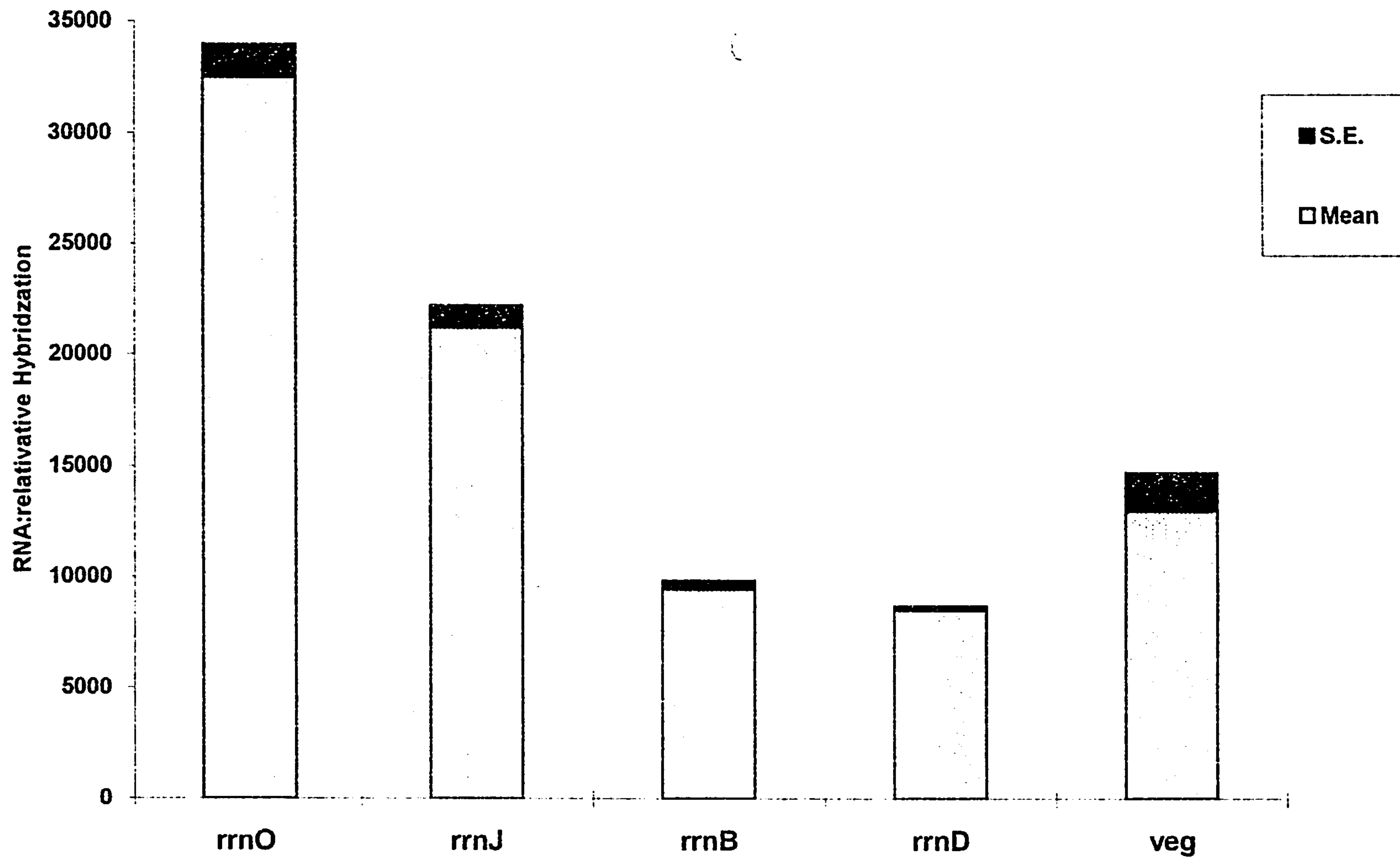


Fig. 6. The relative promoters strength among four *rrn* operons as assayed by dot blot. Total RNA was prepared by a modification of the procedure used for *E. coli* (62), from *B. subtilis* strains IS58 containing integrated *lacZ* fusion:pPW4-*rrnO*, pWAR118-*rrnJ*, pAWR123-*rrnB*, pAWR116-*rrnD* and pPW810-veg (control) respectively. Five and ten micrograms of total RNA from each sample were loaded onto individual slots and hybridized with excess of a *lacZ* specific probe. Conditions for the assay are described in Materials and Methods".



35000
30000
25000
20000
15000
10000
5000
0
RNA:relativative Hybridzation
rrnO
rrnJ
rrnB
rrnD
veg
S.E.
Mean

Fig. 7. The relative strength of individual promoters elements P1 or P2 among four *rrn* operons as assayed by primer extension. Total RNA was prepared by a modification of procedure for *E. coli* (62), from *B. subtilis* strains IS58 containing integrated *lacZ* fusion: pPW4-*rrnO*, pWAR118-*rrnJ*, pAWR123-*rrnB*, pAWR116-*rrnD* and pPW810-*veg* (control) respectively. Primer extension reaction was performed with modifications (63) of a procedure described for *E. coli* (47, 62). The total RNA from 2 ml (75 µg) was mixed with an access of purified 5' labeled primer (8-10 ng). Conditions for the assay are described in Materials and Methods .

The relative strength of individual promoter elements P1 or P2 among four rrn operons and the veg gene as assayed by primer extension

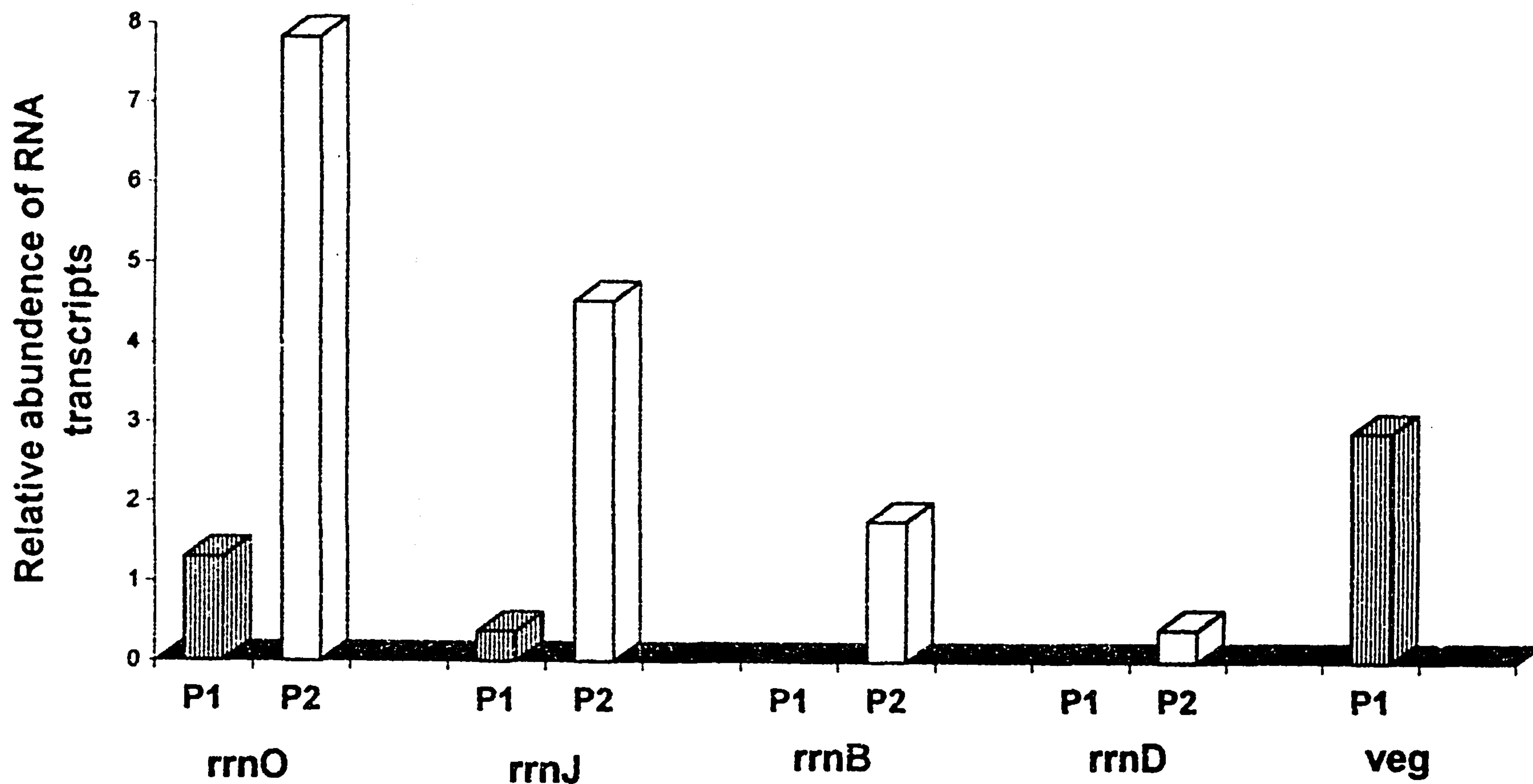


Fig 8. Accumulation of (p)ppGpp of the three isogeneic strains IS58 (lane 1, 2, 3), IS56 (lane 4, 5) and L3 (lane 6, 7) during amino acid starvation. The cell culture was grown in low-phosphate medium to 80 Klett units. The cells were labelled with [^{32}P] phosphoric acid (50- 100 µCi/ml) for one generation. After labelling for 1 hr, the cells were starved for amino acid by the addition of SH to a final concentration of 2 mg/ml. 100 µl samples were taken after 0, 5 and 15 min in the case of IS58, 0 and 15 in the case of IS56 and 0, 5 and 15 min for L3 respectively. The samples mixed with 13 M formic acid and frozen. After thawing, samples were centrifuged and 10 µl of the supernatant were applied to polyethyleneimine-cellulose (PEI) plates for thin-layer chromatography of the phosphorylated guanosine nucleotides in 1.5 M KH_2PO_4.

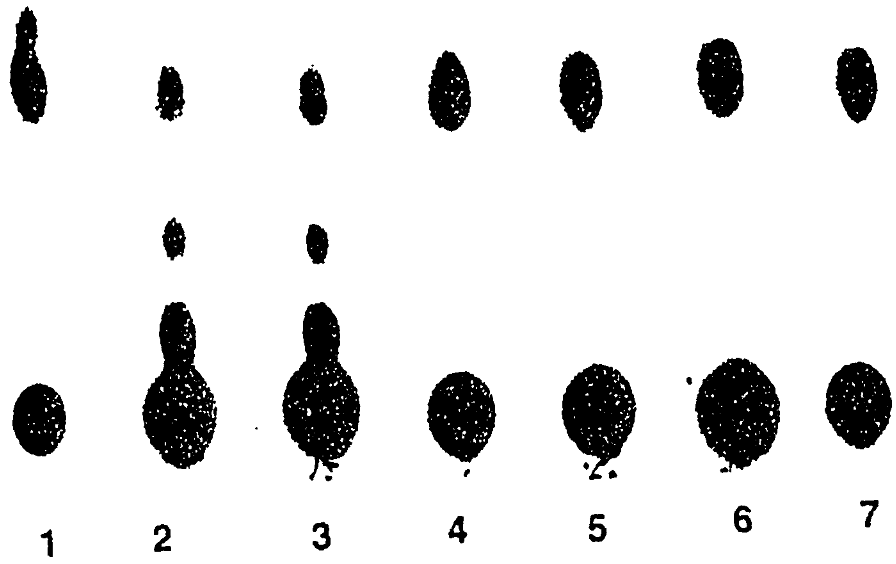
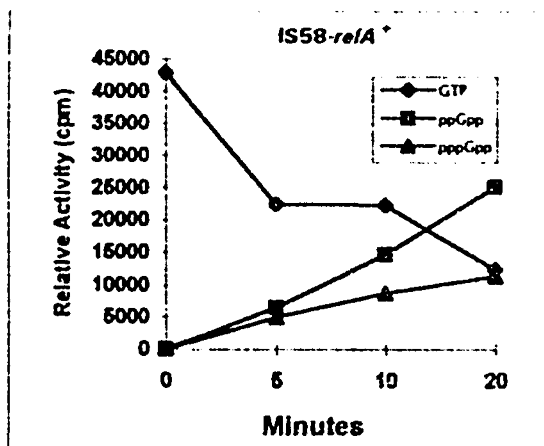
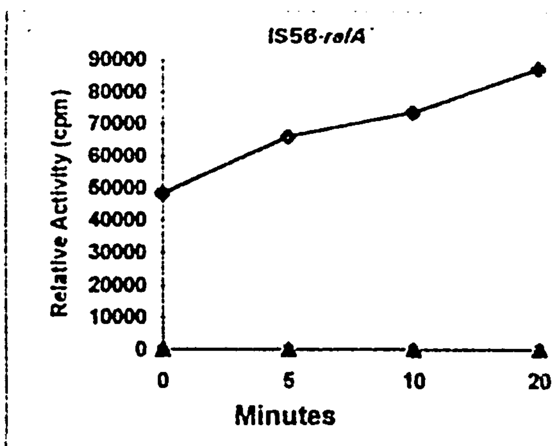

GTP
ppGpp
pppGpp
ORIGIN
1 2 3 4 5 6 7

Fig. 9. Plot of the accumulation of (p)ppGpp during treatment of *B. subtilis* strains (IS58, IS56 and L3) with SH based on densitometry. Samples were taken from IS58 after 0, 5, 10, and 20 min, from IS56 after 0, 5, 10, and 20 min, and from L3 after 0, and 15 min. This plot does not reflect the photograph shown in figure 8. See Materials and Methods for details.


IS58-relA+
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Relative Activity (cpm)
GTP
ppGpp
pppGpp
0
5
10
20
Minutes


IS56-relA-
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Relative Activity (cpm)
0
5
10
20
Minutes

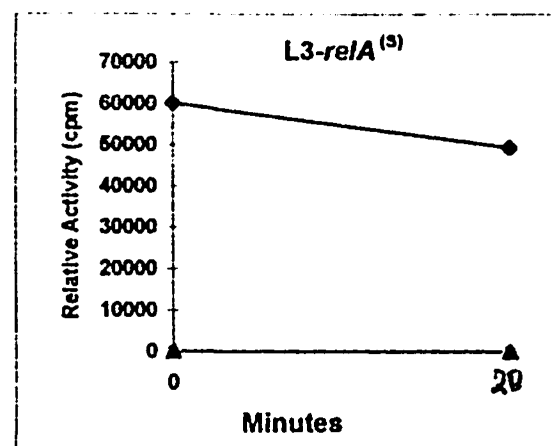

L3-relA(S)
70000
60000
50000
40000
30000
20000
10000
0
Relative Activity (cpm)
0
20
Minutes

Fig 10. Accumulation of (p)ppGpp in all of the three isogeneic strains IS58 (lane 1, 2, 3) , IS56 (lane 4, 5) and L3 (lane 6, 7, 8) during carbon source limitation. The cell culture was grown in low-phosphate medium to 80 Klett reading. The cells were labelled with [^{32}P] phosphoric acid (50- 100 μCi/ml) for one generation. After labelling for 1 hr, the cells were starved for carbon source by the addition of αMG to a final concentration of 1%. Samples (100 μl) were taken after 0, 10 and 15 min in the case of IS58, 0, 15 in the case of IS56 and 0, 15 for L3 respectively. The samples mixed with 13 M formic acid and frozen. After thawing, samples were centrifuged and 10 μl of the supernatant were applied to polyethyleneimine-cellulose (PEI) plates for thin-layer chromatography of the phosphorylated guanosine nucleotides in 1.5 M KH_2PO_4.

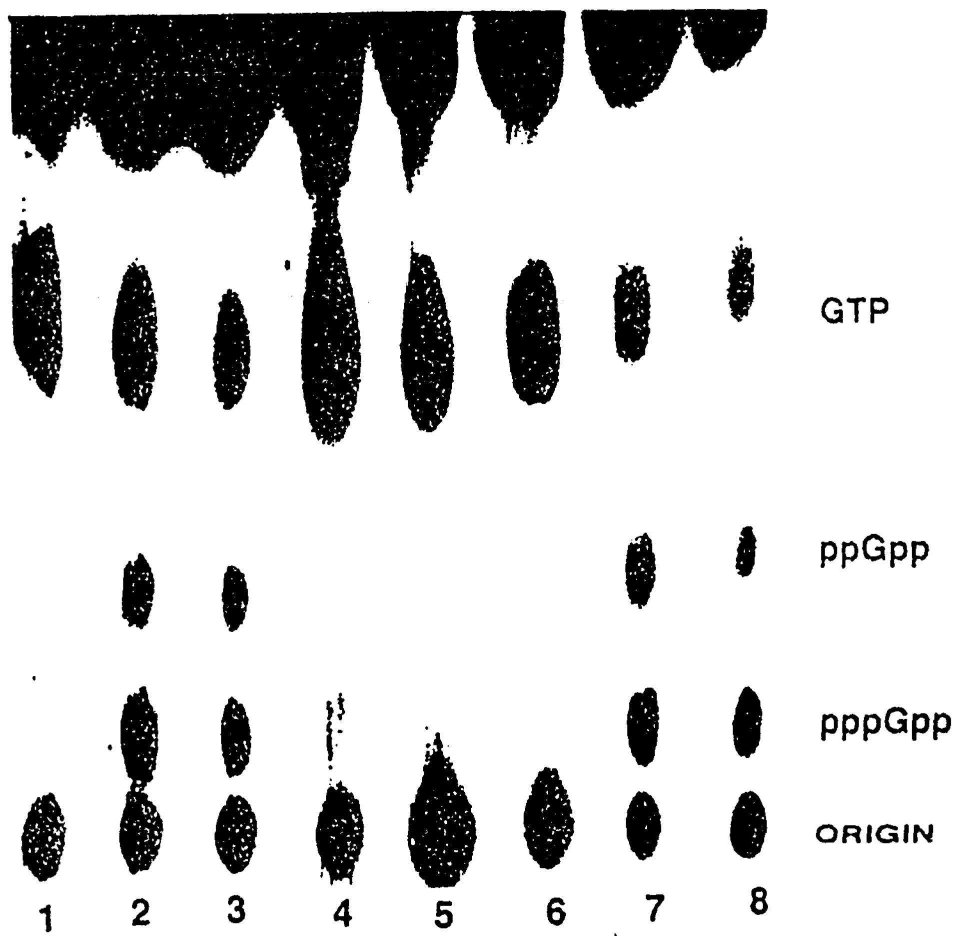
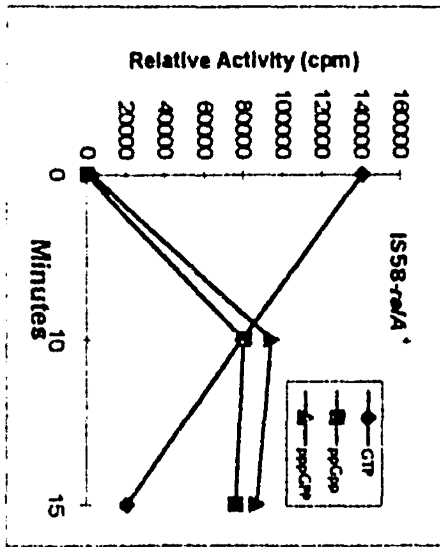
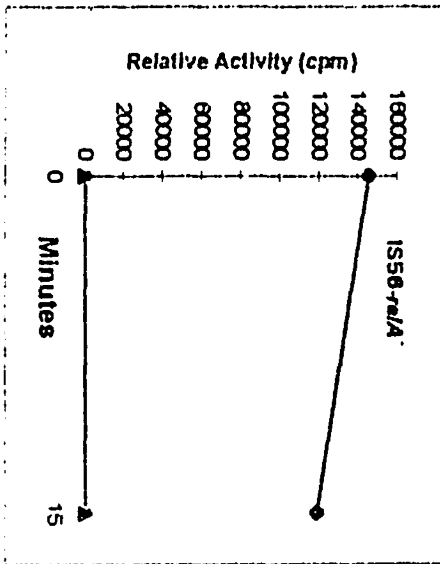

GTP
ppGpp
pppGpp
ORIGIN
1
2
3
4
5
6
7
8

Fig. 11. Plot of the accumulation of (p)ppGpp during treatment of *B. subtilis* strains (IS58, IS56 and L3) with αMG based on densitometry. Samples were taken from IS58 after 0, 10, and 15 min, from IS56 after 0, and 15 min, and from L3 after 0, 10, and 15 min. This plot does not reflect the photograph shown in figure 10. See materials and methods for details.


IS58-relA
Relative Activity (cpm)
Minutes
GTP
ppGpp
pppGpp


Relative Activity (cpm)
Minutes

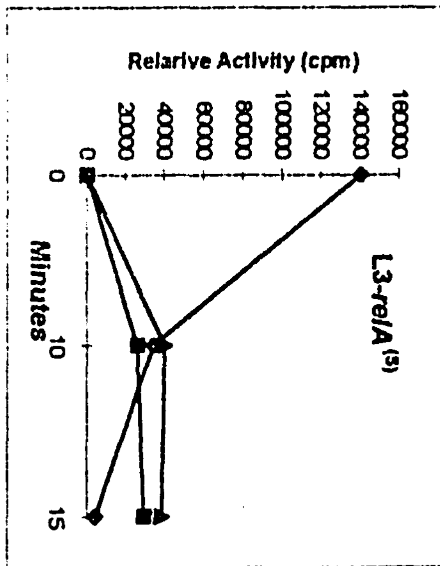
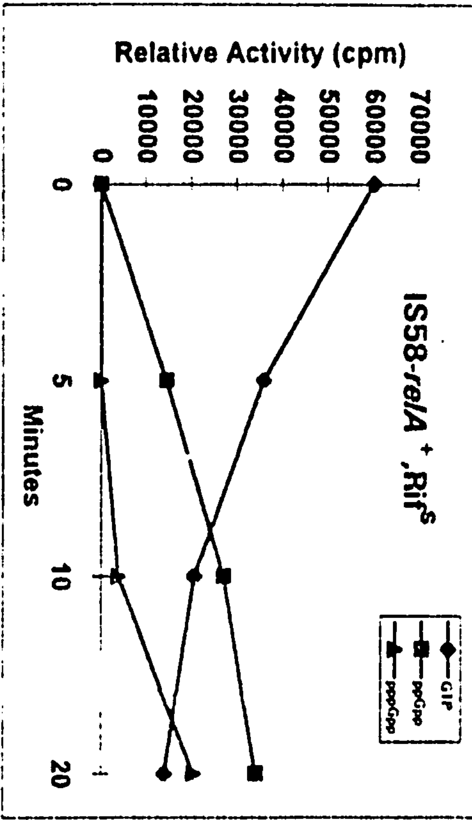
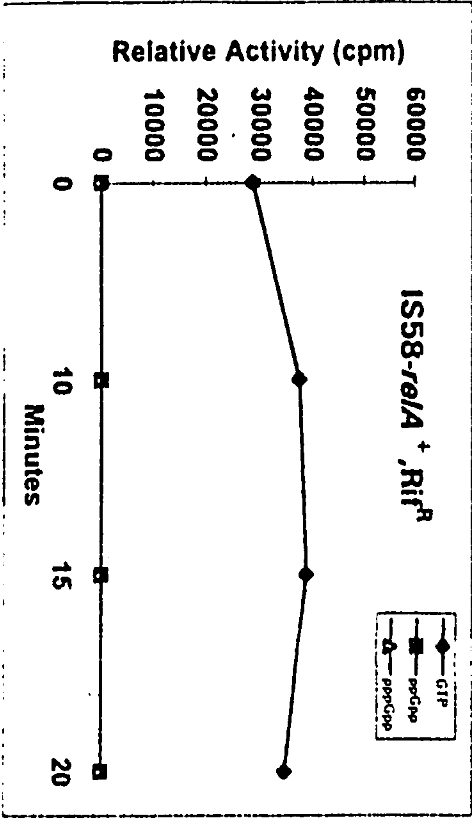
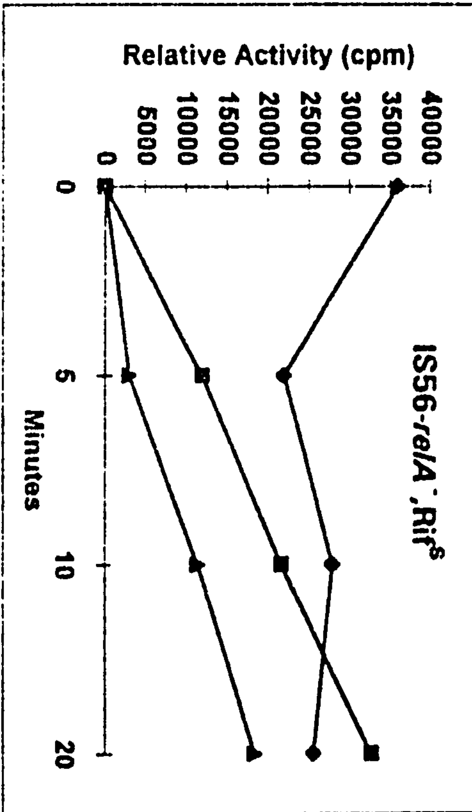

L3-relA
Relative Activity (cpm)
Minutes

Fig. 12. The Plot of the accumulation of (p)ppGpp of *B. subtilis* strains during rifampin treatment. The *B. subtilis* strains used are IS58 (*trpC2, lys-3, relA*$^+$) , IS56 (*trpC2, lys-3, relA*$^-$), ISR58 (*trpC2, lys-3, relA*$^+$*, rpoB18*), and ISR56 (*trpC2, lys-3, relA*$^-$*, rpoB18*). The cell culture was grown in low-phosphate medium to a reading of 80 Klett units. The cells were labelled with [^{32}P] phosphoric acid (50- 100 µCi/ml) for one generation. After labelling for 1 hr, the cells were treated with 10 µg/ml rifampin. Samples (100 µl) were taken from IS58 and IS56 after 0, 5, 10 and 20 min. Samples (100 µl) were taken from ISR58 and ISR56 after 0, 10, 15 and 20 min. The samples were mixed with 13 M formic acid and frozen. After thawing, samples were centrifuged and 10 µl of the supernatant were applied to polyethyleneimine-cellulose (PEI) plates for thin-layer chromatography of the phosphorylated guanosine nucleotides in 1.5 M KH_2PO_4.


IS58-relA +, RifS
Relative Activity (cpm)
0
10000
20000
30000
40000
50000
60000
70000
Minutes
0
5
10
20
GTP
ppGpp
pppGpp


IS58-relA +, RifR
Relative Activity (cpm)
0
10000
20000
30000
40000
50000
60000
Minutes
0
10
15
20
GTP
ppGpp
pppGpp


IS56-relA -, RifS
Relative Activity (cpm)
0
5000
10000
15000
20000
25000
30000
35000
40000
Minutes
0
5
10
20

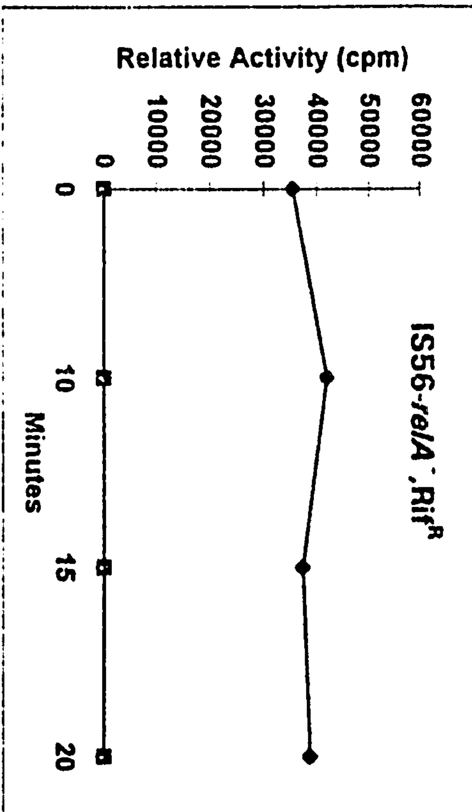
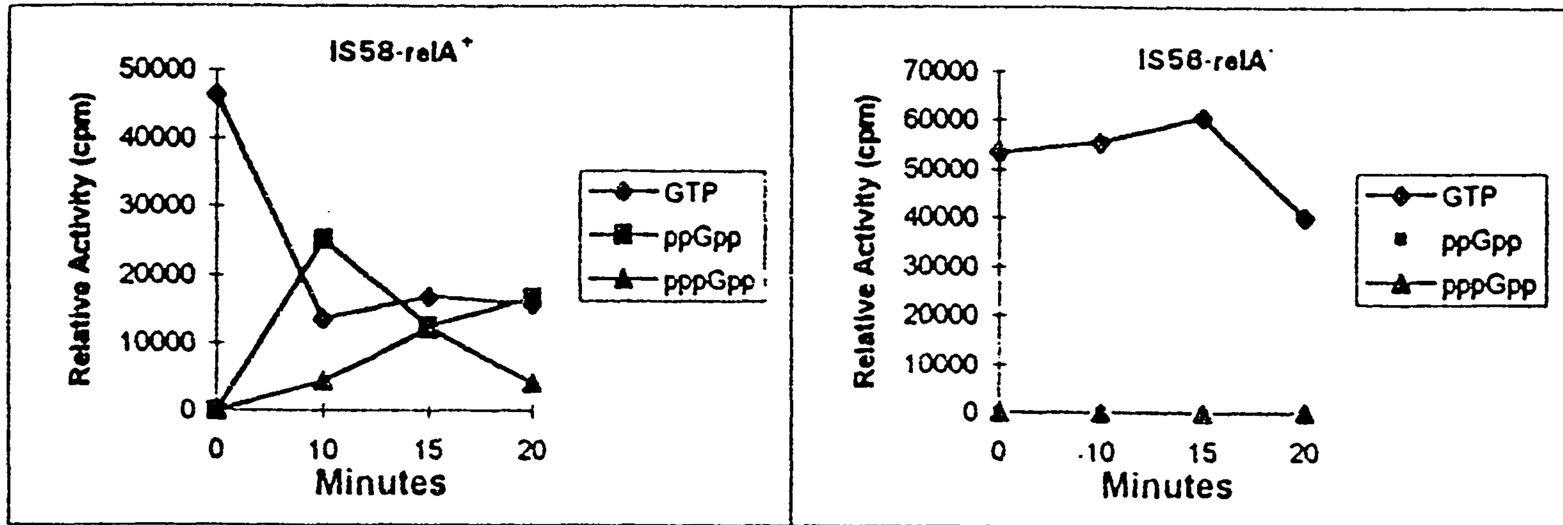

IS56-relA -, RifR
Relative Activity (cpm)
0
10000
20000
30000
40000
50000
60000
Minutes
0
10
15
20

Fig. 13. Accumulation of (p)ppGpp during treatment of *B. subtilis* strains (ISR58 and ISR56) with SH based on densitometry tracing.



IS58-relA+
Relative Activity (cpm)
50000
40000
30000
20000
10000
0
0
10
15
20
Minutes
GTP
ppGpp
pppGpp
IS58-relA-
Relative Activity (cpm)
70000
60000
50000
40000
30000
20000
10000
0
0
10
15
20
Minutes
GTP
ppGpp
pppGpp

Fig. 14. The effect of serine hydroxamate on the expression of the *rrnO-lacZ* fusion of *rrnO* tandem promoters and promoter elements (pPW4, pLR501, pLR512) in *B. subtilis relA* wild-type and mutant strains (IS58, IS56) as assayed by β- galactosidase (see Table 2A, 4 and 5). The cell cultures were grown in MM1 medium. 1 ml samples were taken at 0, 30, 60 min after SH treatment and assayed as described in Materials and Methods.

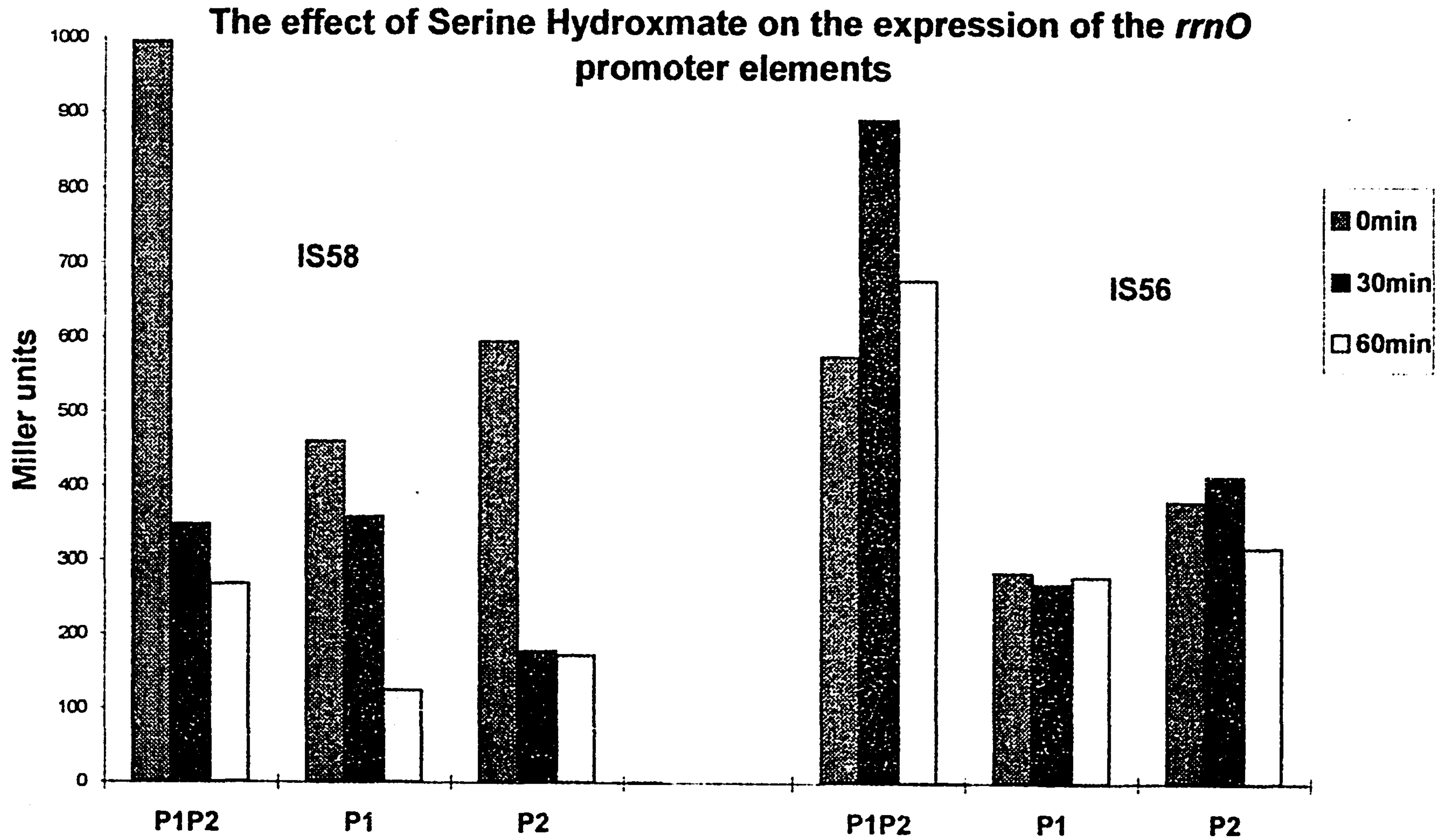

The effect of Serine Hydroxmate on the expression of the *rrnO* promoter elements
Miller units
1000
900
800
700
600
500
400
300
200
100
0
IS58
IS56
0min
30min
60min
P1P2
P1
P2
P1P2
P1
P2

Fig 15. The effect of serine hydroxamate on the expression of the *rrnJ-lacZ* fusion of *rrnJ* tandem promoters and promoter elements (pAWR118, pLR105, pLR210) in *B. subtilis relA* wild-type and mutant strains (IS58, IS56) as assayed by β- galactosidase (see Table 2A, 4 and 5). The cell cultures were grown in MM1 medium. Samples of 1 ml were taken at 0, 30, 60 min after SH treatment, then the Miller units were calculated.

The effect of Serine Hydroxymate on the expression of *rrnJ* promoter elements

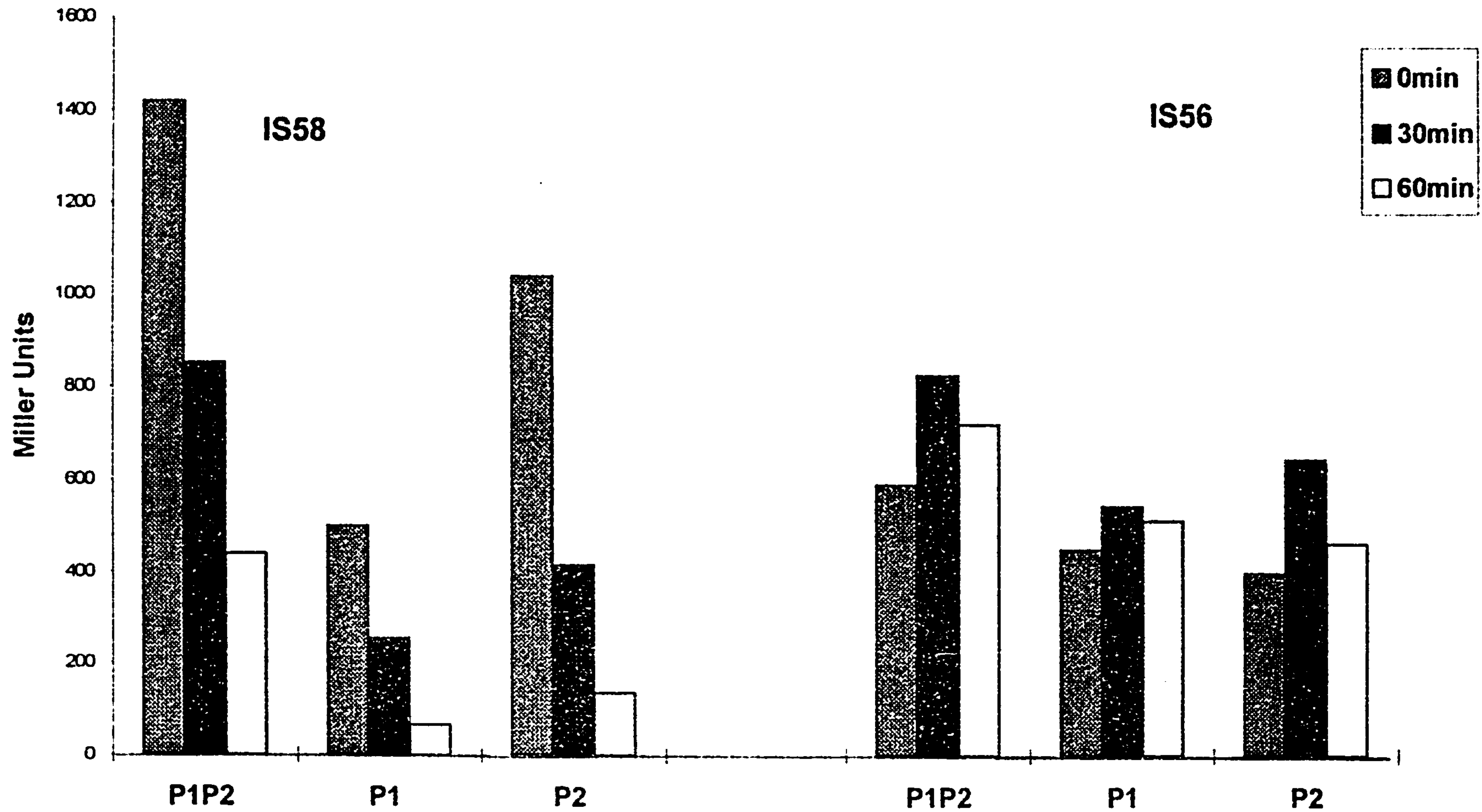


Fig 16. The effect of serine hydroxamate on the expression of *rrnO-lacZ* fusion of the tandem promoters and the promoter elements (pPW4, pLR501, pLR512) in IS58 and IS56 strains as assayed by RNA dot blot. Cell cultures grown in MM1 medium. 5 and 10 µg/ml samples at 0 and 60 min, were loaded onto nitrocellulose inserted in slot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determine by hybridization to probe specific for *lacZ* message. The probe was labelled with the random primer extension kit, using [α-^{32}P]dCTP. An example of dot blot shown below. Results are the average of three independent mRNA isolations.

The Effect of Serine Hydroxamate on the Expression of rrnO Promoter as Assayed by RNA Dot Blot

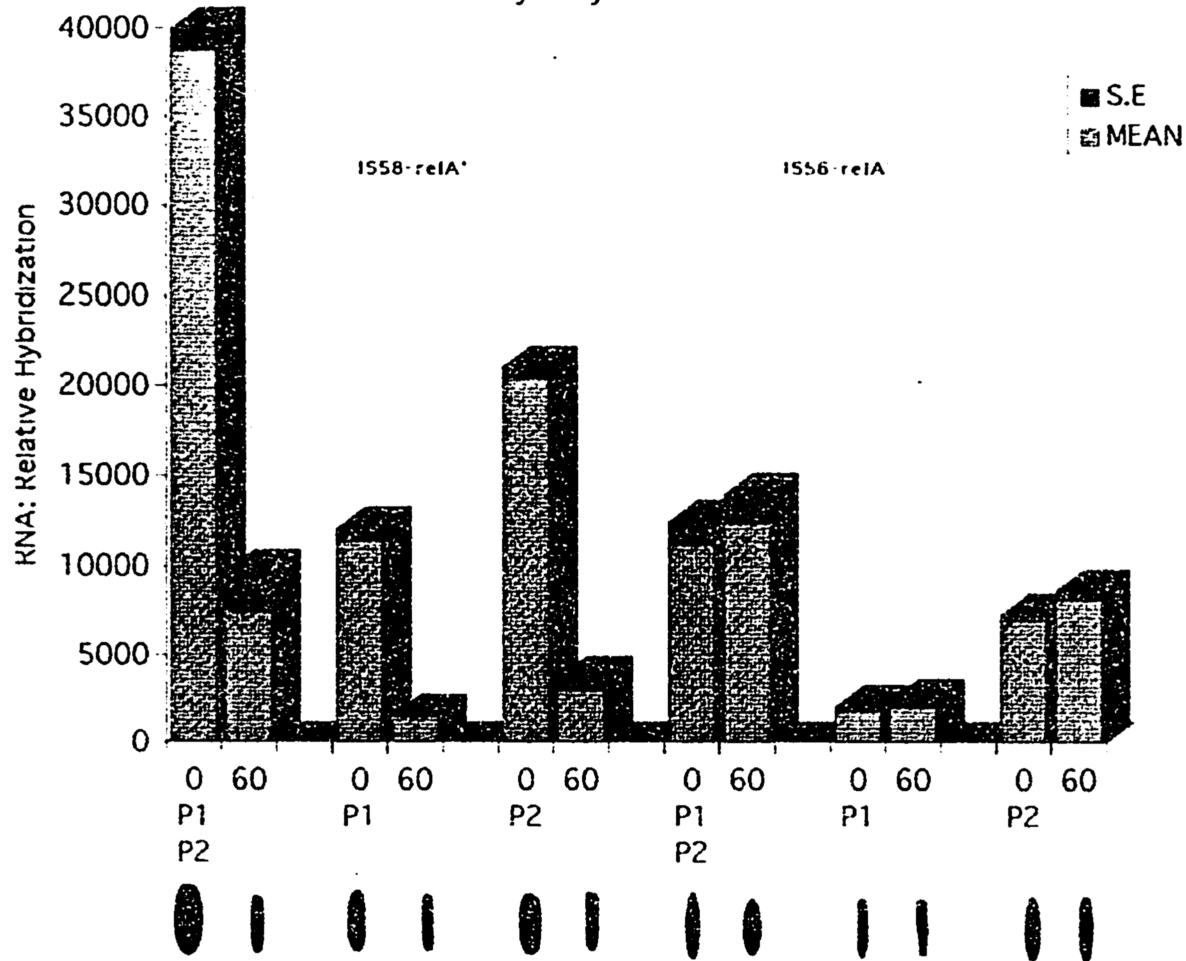


Fig. 17. The effect of serine hydroxamate on the expression of *rrnJ-lacZ* fusion of the tandem promoters and the promoter elements (pAWR118, pLR105, pLR210) in IS58 and IS56 strains as assayed by RNA dot blot. Cell cultures grown in MM1 medium. 5-10 µg/ml samples at 0 and 60 min, were loaded onto nitrocellulose inserted in slot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determine by hybridization to probe specific for lacZ message. The probe was labelled with the random primer extension kit, using [α-^{32}P]dCTP. Results are the average of three independent mRNA isolations.

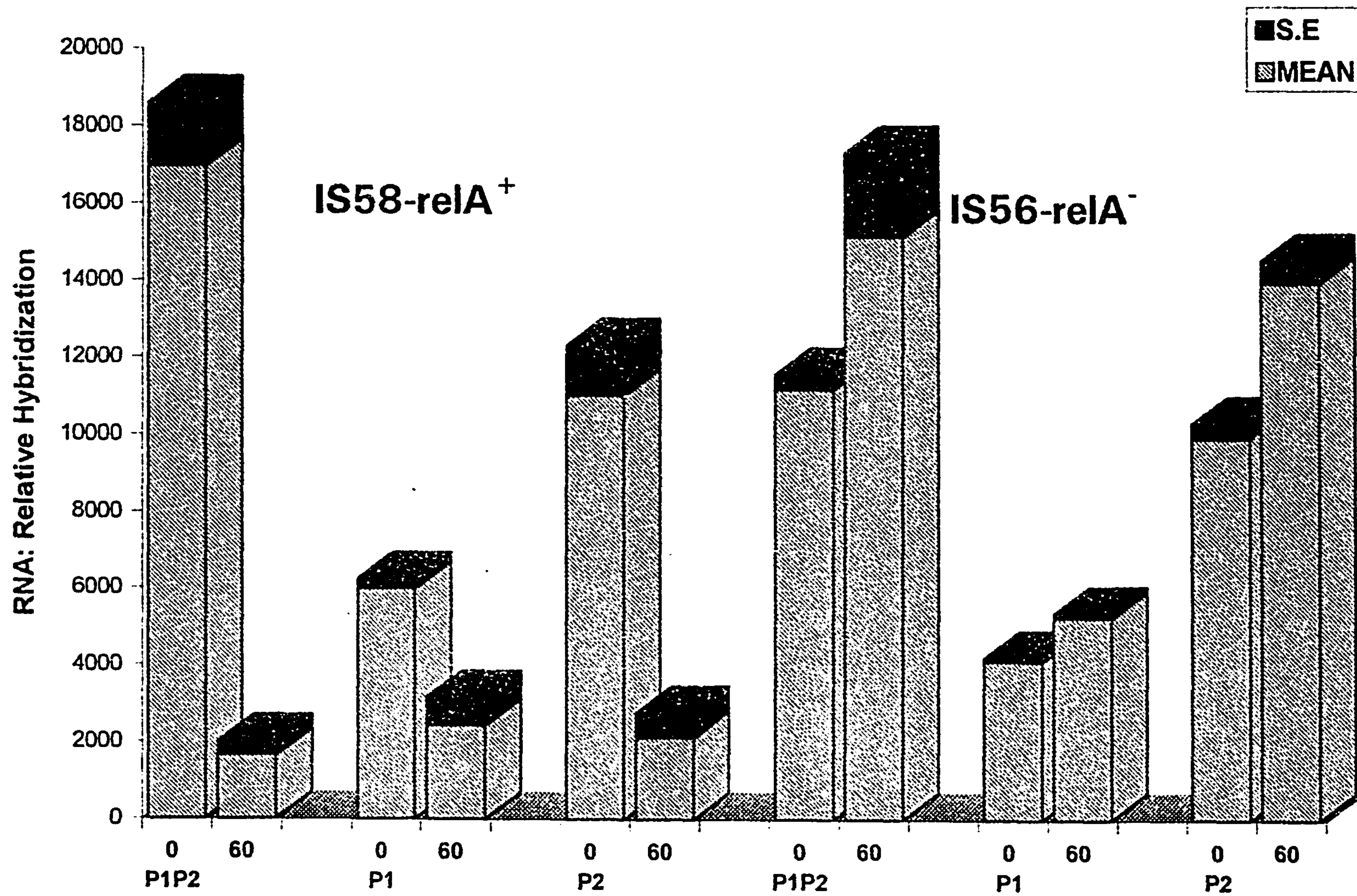

S.E
MEAN
RNA: Relative Hybridization
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
IS58-relA^{+}
IS56-relA^{-}
0
60
P1P2
0
60
P1
0
60
P2
0
60
P1P2
0
60
P1
0
60
P2

Fig. 18. The effect of serine hydroxmate on the expression of *rrn-lacZ* fusion of pPW4-*rrnO*, pAWR108-*rrnJ*, pAWR118-*rrnB*, pAWR116-*rrnD*, and pAWR810-*veg* (control), in IS58 strains as assayed by RNA dot blot. Cell cultures grown in MM1 medium. 5 and 10 µg/ml samples at 0 and 60 min, were loaded onto nitrocellulose inserted in slot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determine by hybridization to probe specific for lacZ message. The probe was labelled with the random primer extension kit, using [α-^{32}P]dCTP.

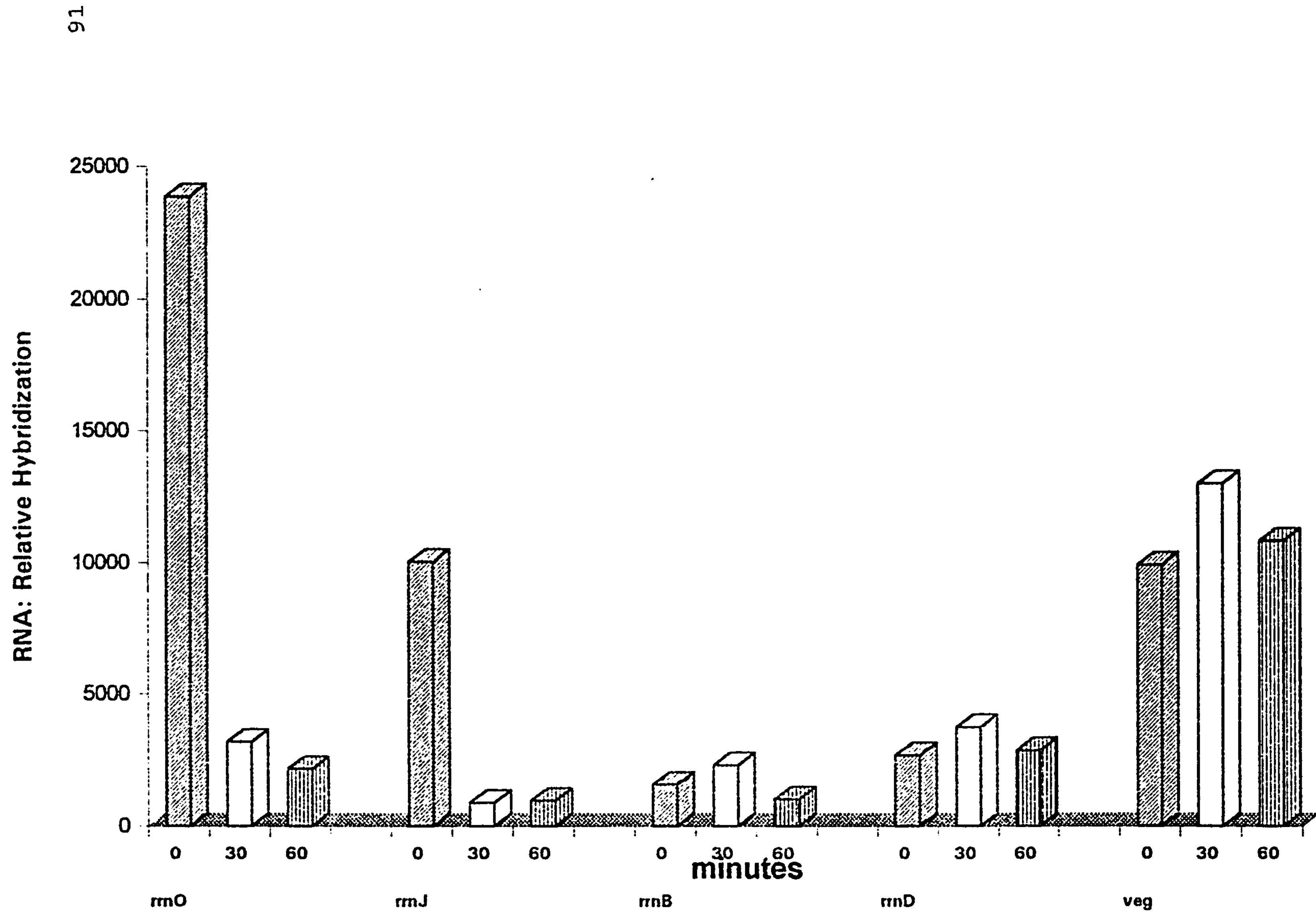

RNA: Relative Hybridization
25000
20000
15000
10000
5000
0
0
30
60
0
30
60
0
30
60
0
30
60
0
30
60
minutes
rrnO
rrnJ
rrnB
rrnD
veg

Fig 19. The effect of Alpha methyl glucoside on the expression of *rrnO-lacZ* fusion of the tandem promoters and the promoter elements (pPW4, pLR501, pLR512) in IS58 and IS56 strains as assayed by RNA dot blot. Cell cultures were grown in MM1 medium. 5 and 10 µg/ml samples at 0 and 90 min, were loaded onto nitrocellulose inserted in the dot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determine by hybridization to probe specific for lacZ message. The prob was labelled with the random primer extension kit, using [α-^{32}P]dCTP. An example of dot blot shown below. Results are the average of three independent mRNA isolations.

The Effect of Alpha Methyl on the Expression of rrnO Promoter as Assayed by RNA Dot Blot

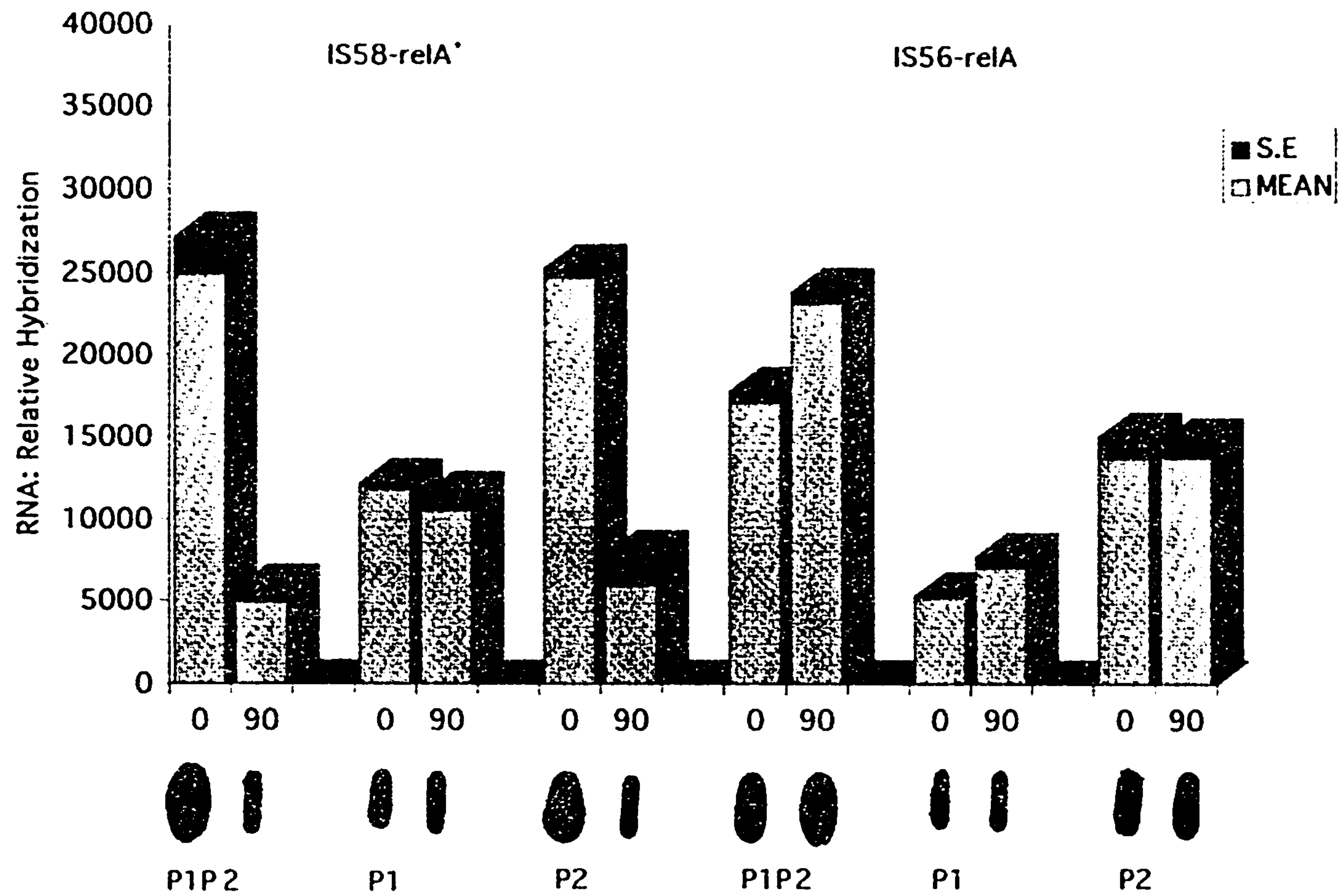


Fig. 20. The effect of α alpha methyl glucoside on the expression of *rrnJ-lacZ* fusion of the tandem promoters and the promoter elements (pPW4, pLR501, pLR512) in IS58 and IS56 strains as assayed by RNA slot blot. Cell cultures were grown in MM1 medium. 5 and 10 μg/ml samples at 0 and 90 min, were loaded onto nitrocellulose inserted in dot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determined by hybridization to a probe specific for lacZ message. The probe was labelled with the random primer extension kit, using [α-^{32}P]dCTP.

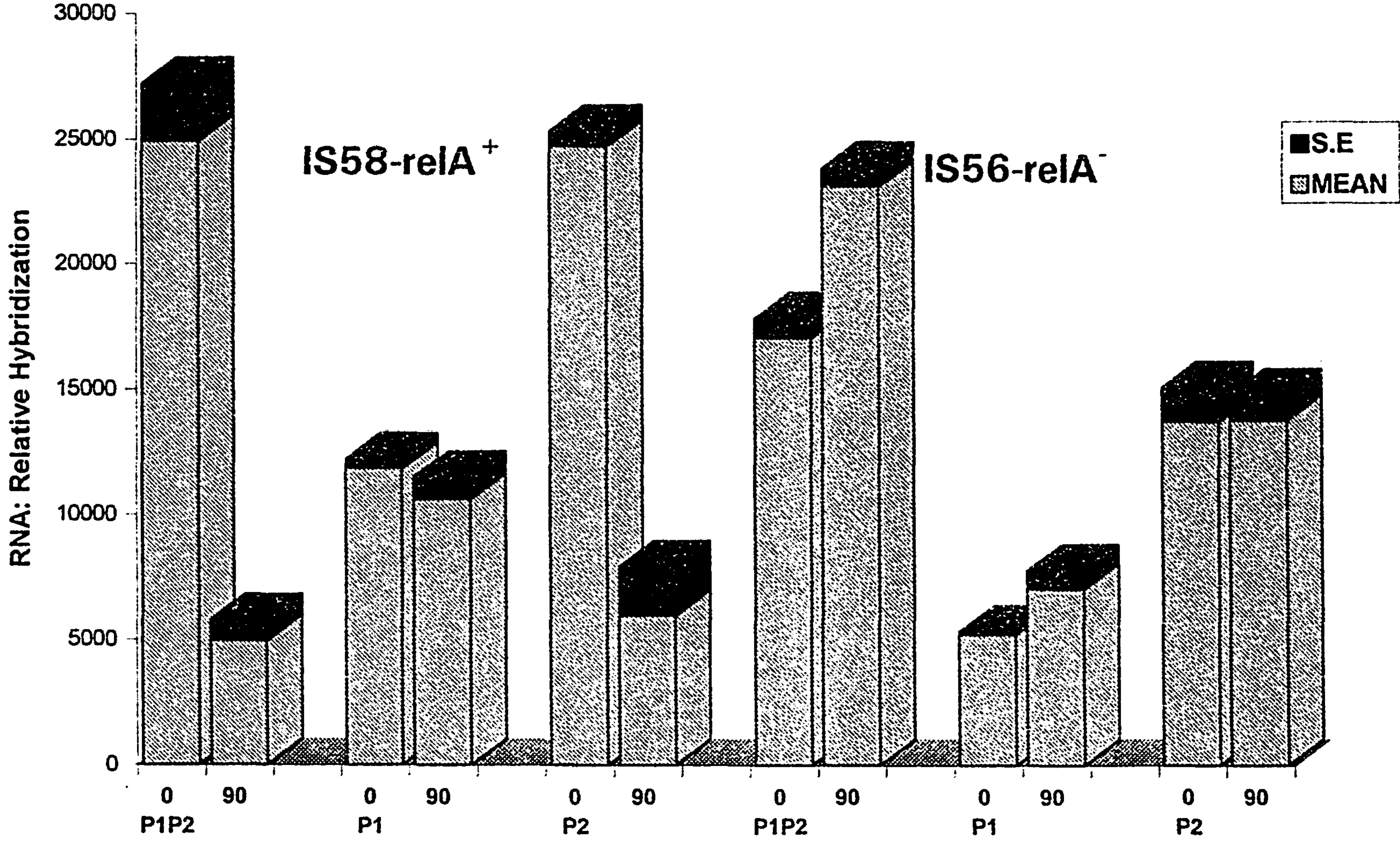

RNA: Relative Hybridization
30000
25000
20000
15000
10000
5000
0
IS58-relA+
IS56-relA-
S.E
MEAN
0
90
P1P2
0
90
P1
0
90
P2
0
90
P1P2
0
90
P1
0
90
P2

Fig. 21 The effect of α methyl glucoside on the expression of *rrn- lacZ* fusion of pPW4-*rrnO*, pWAR118-*rrnJ*, pAWR123-*rrnB*, pAWR116-*rrnD*, and pAWR810-*veg* (control), in IS58 strains as assayed by RNA dot blot. Cell cultures were grown in MM1 medium. 5 and 10 µg/ml samples at 0 and 60 min, were loaded onto nitrocellulose inserted in slot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determine by hybridization to probe specific for lacZ message. The probe was labelled with the random primer extension kit, using [α-^{32}P]dCTP.

RNA: Relative Hybridization

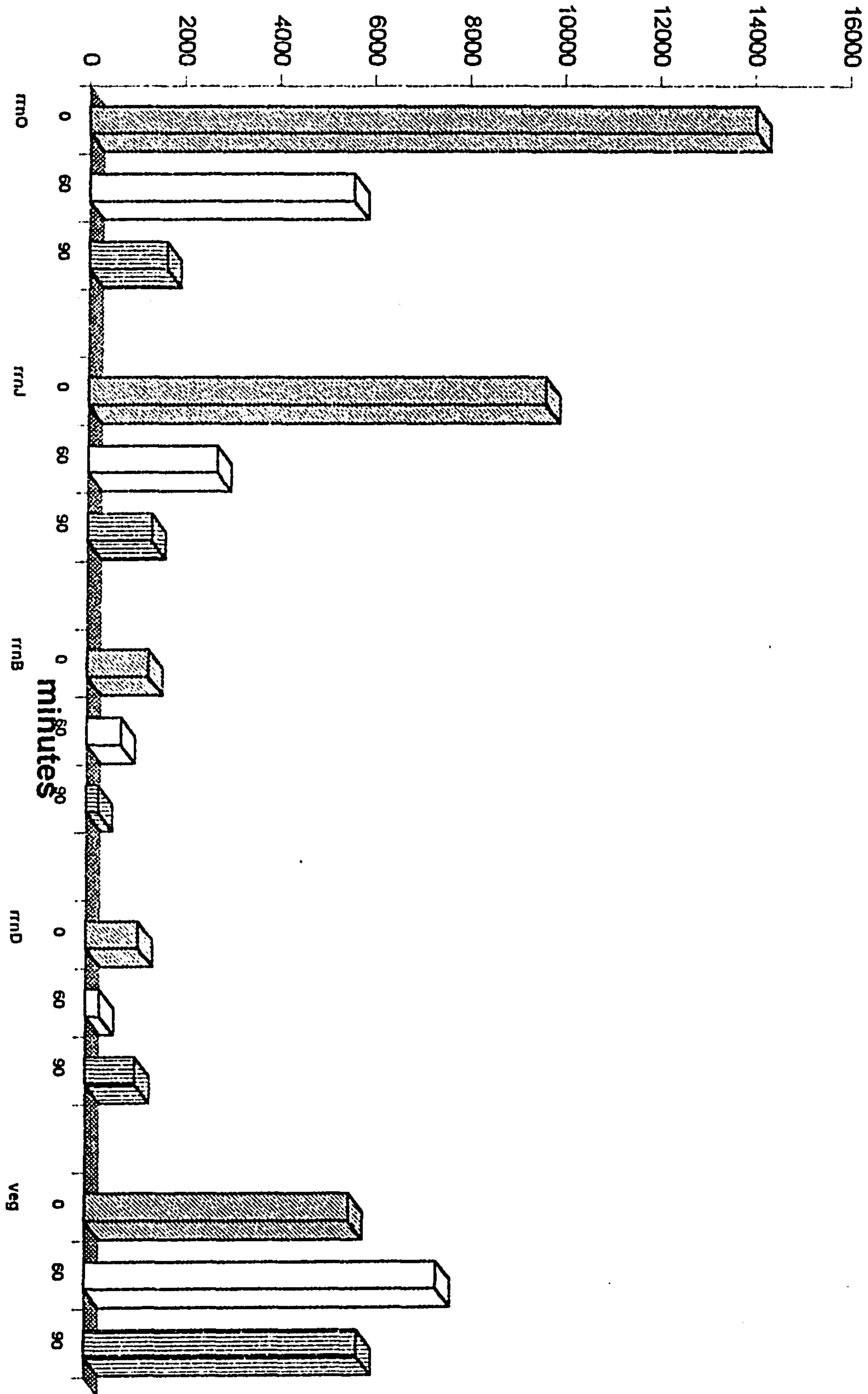


Fig. 22 Primer extension analysis of *rrnO* transcripts. The RNA was isolated from *B. subtilis* cultures treated with two different treatments (SH and αMG). The *B. subtilis* strains treated with SH (2 mg/ml) are IS58 (lanes 1, 2) , L3 (lanes 3, 4) and IS56 (lanes 5, 6). The *B. subtilis* strains treated with αMG (1%) are IS58 (7, 8), L3 (9 ,10) and IS56 (11, 12). RNA samples were taken at intervals 0, 60 min in the case of SH treatment and 0, 90 min in the case αMG treatment. A radiolabeled oligonucleotide with ^{32}P-γ-ATP (5'- TGC AGG CCC TAG TTT GAC TGA CTA C 3' complimentary to the unique sequence of *rrnO* at -256 to -221) was used to prime DNA synthesis for the RNA template as described in material and methods. The sizes of the two products resolved are P1 150 bp and P2 39 bp.

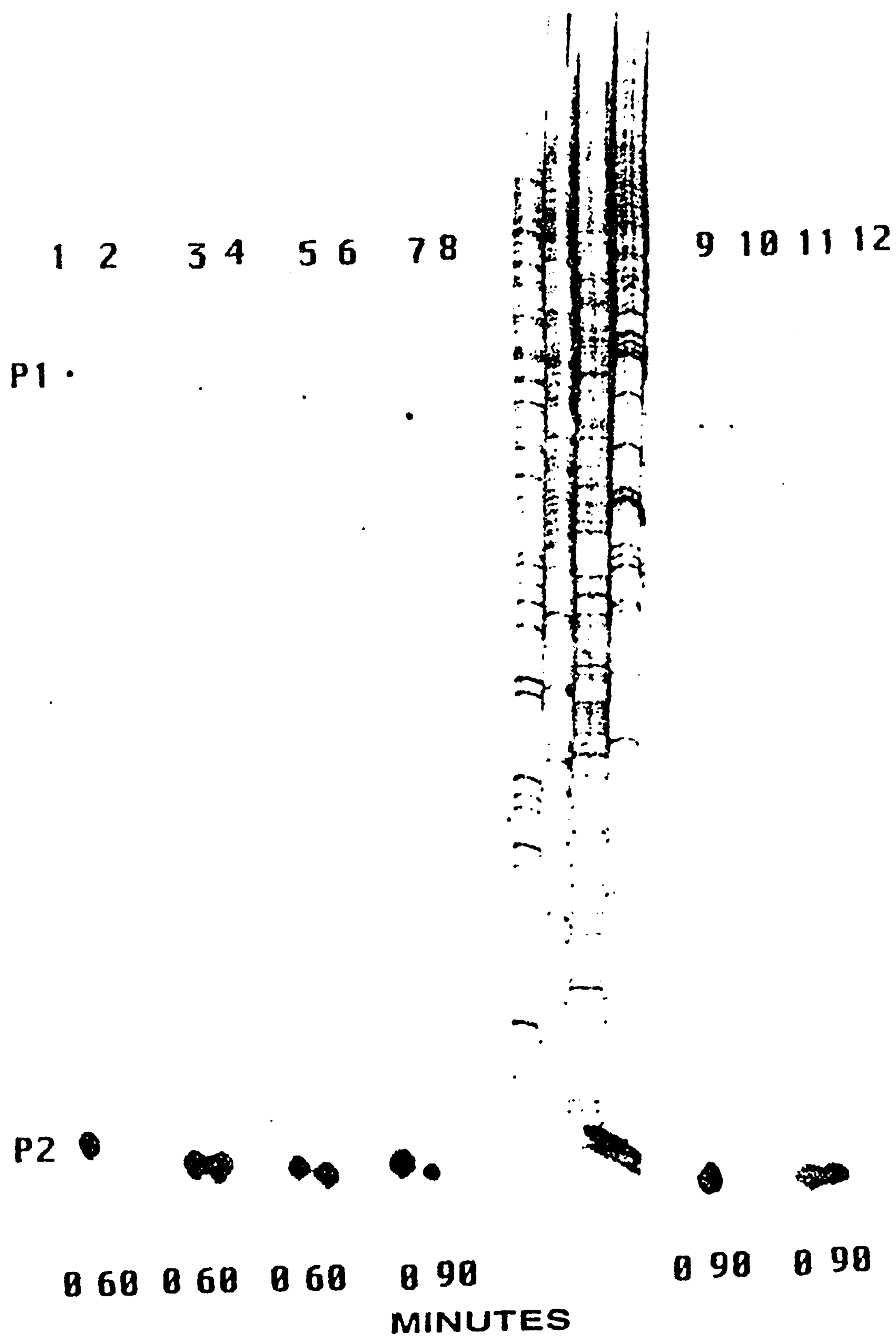

1 2 3 4 5 6 7 8
9 10 11 12
P1
P2
0 60 0 60 0 60 0 90
0 90 0 90
MINUTES

Fig. 23 Quantitation of primer extension analysis of *rrnO* transcripts. RNA was isolated from *B. subtilis* IS58, IS56 and L3 at intervals 0, 60 min after the addition of SH (2 mg/ml). Quantitation of primer extension products was done by densitometry. See Figure 22 and materials and methods for further details.

The Effect of Serine Hydroxamate on the Expression of of rrnO Promoters P2 and P2 as Assayed by Primer Extension Analysis

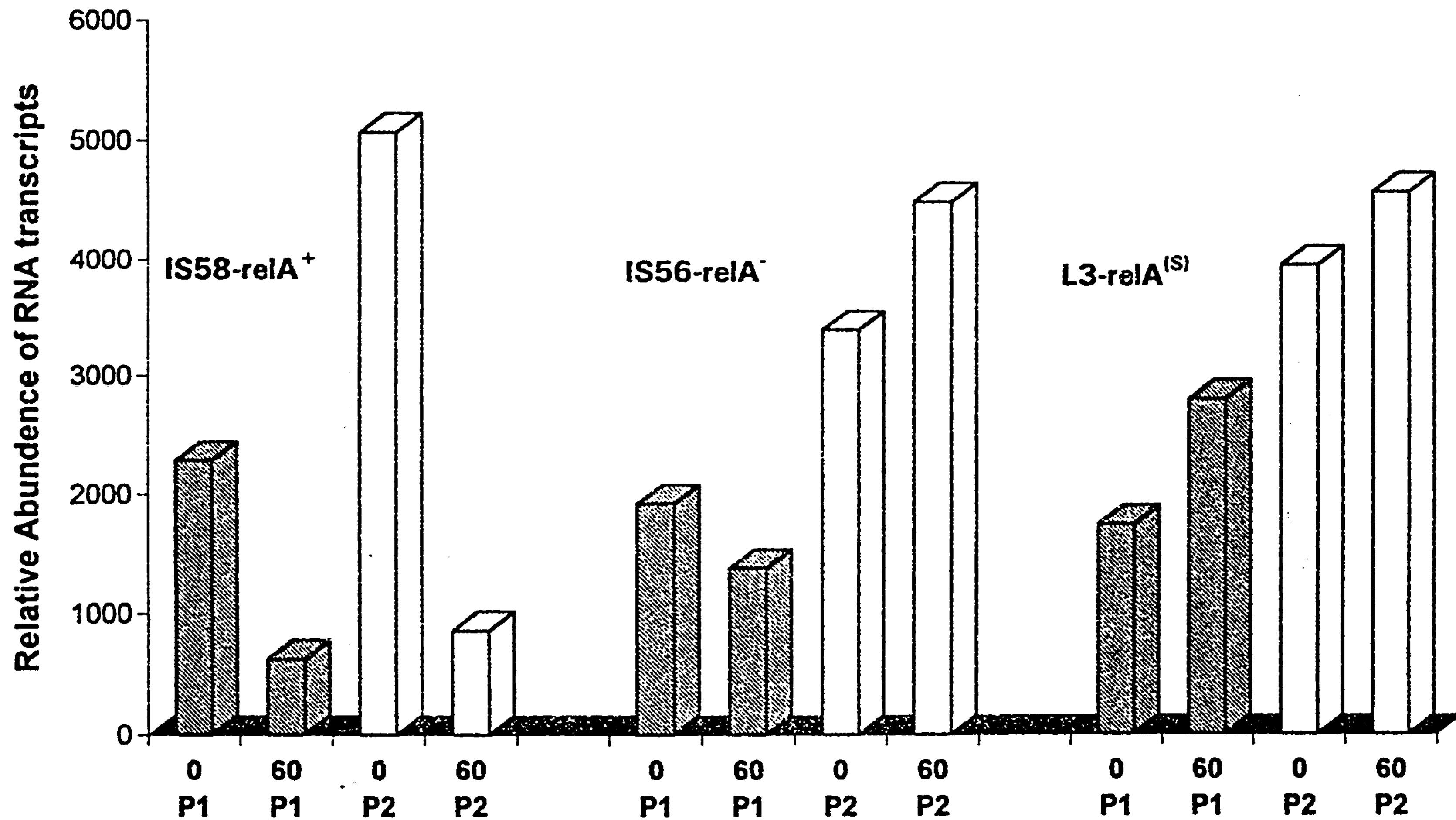


Fig. 24 Quantitation of primer extension analysis of *rrnO* transcripts. RNA was isolated from *B. subtilis* IS58, IS56 and L3 at intervals 0, 90 min after the addition of αMG (1%). Quantitation of primer extension products was done by densitometry. See Figure 22 and materials and methods for further details.

The Effect of Alpha Methyl Glucoside on the Expression rrnO Promoters P1 and P2 as Assayed by Primer Extension Analysis

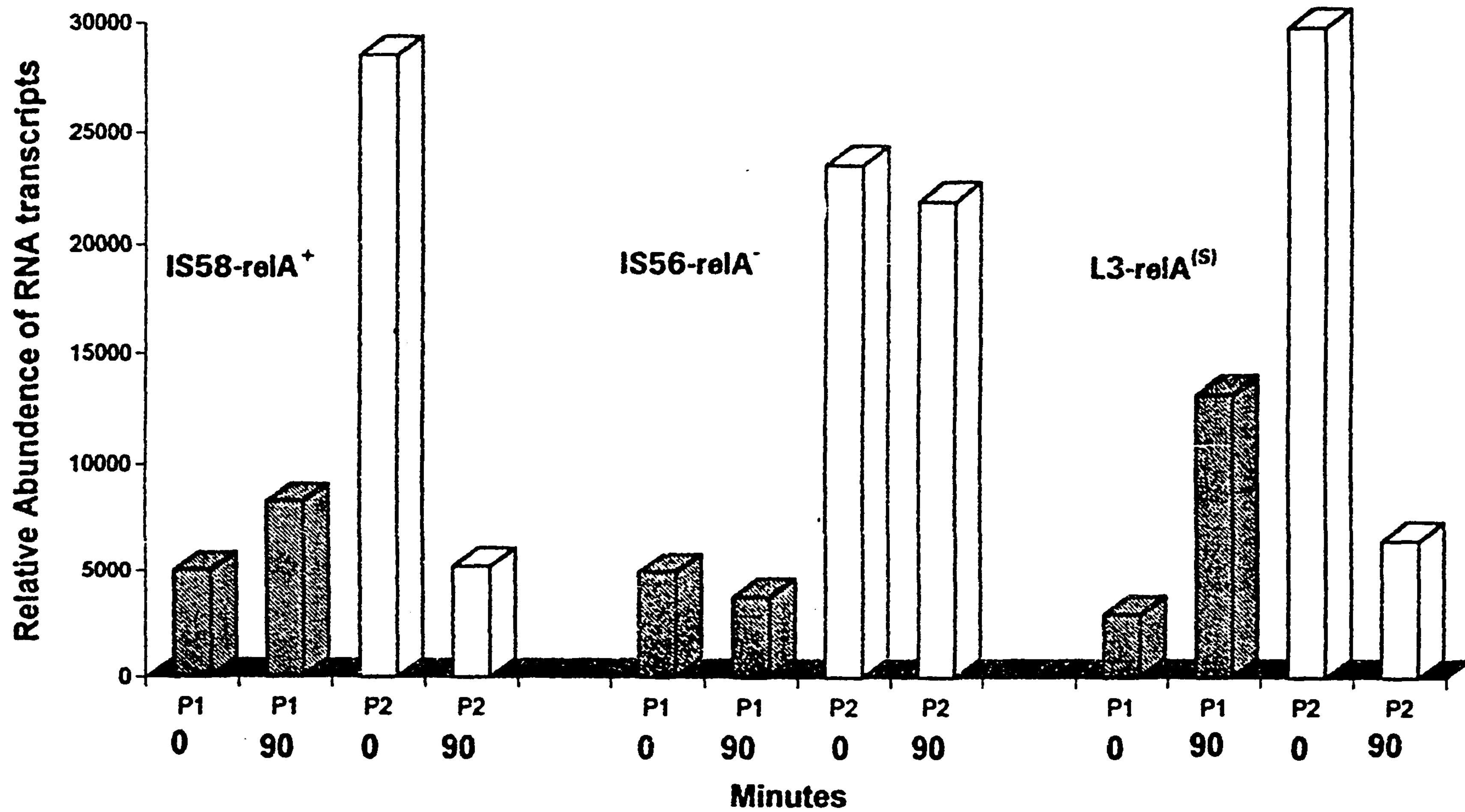


Fig. 25 Quantitation of primer extension analysis of *rrnO* transcripts. RNA was isolated from *B. subtilis* IS58 and IS56 at intervals 0, 5 min after the addition of rifampin (10 μg/ml). A radiolabeled oligonucleotide with ^{32}P-γ-ATP (5'- TGC AGG CCC TAG TTT GAC TGA CTA C 3' complimentary to the unique sequence of *rrnO* at -256 to -221) was used to prime DNA synthesis for the RNA template as described in material and methods. Quantitation of primer extension products was done by densitometry.

The Effect of Rifampin on the Expression of rrnO Promoter as Assayed by Primer Extension Analysis

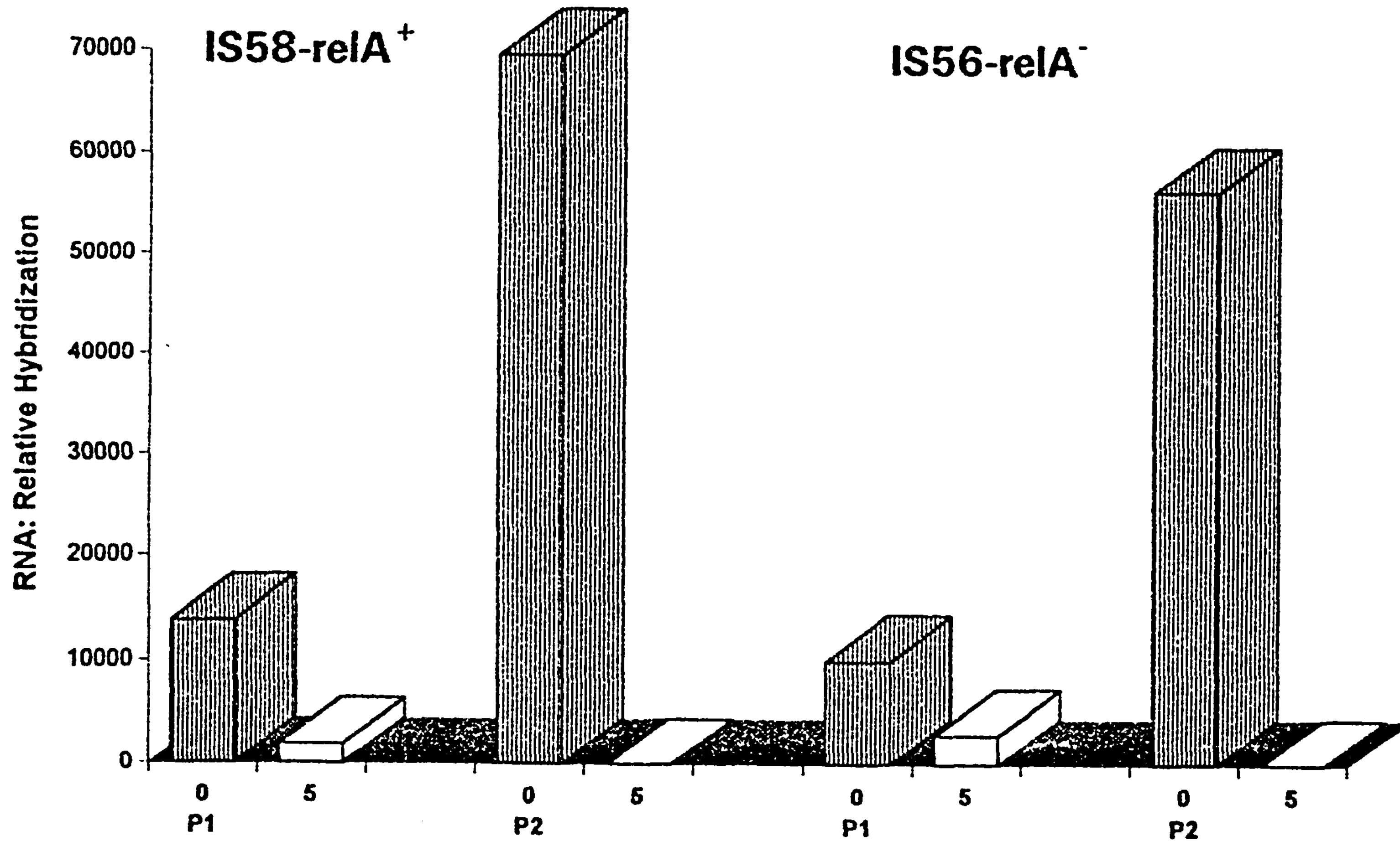
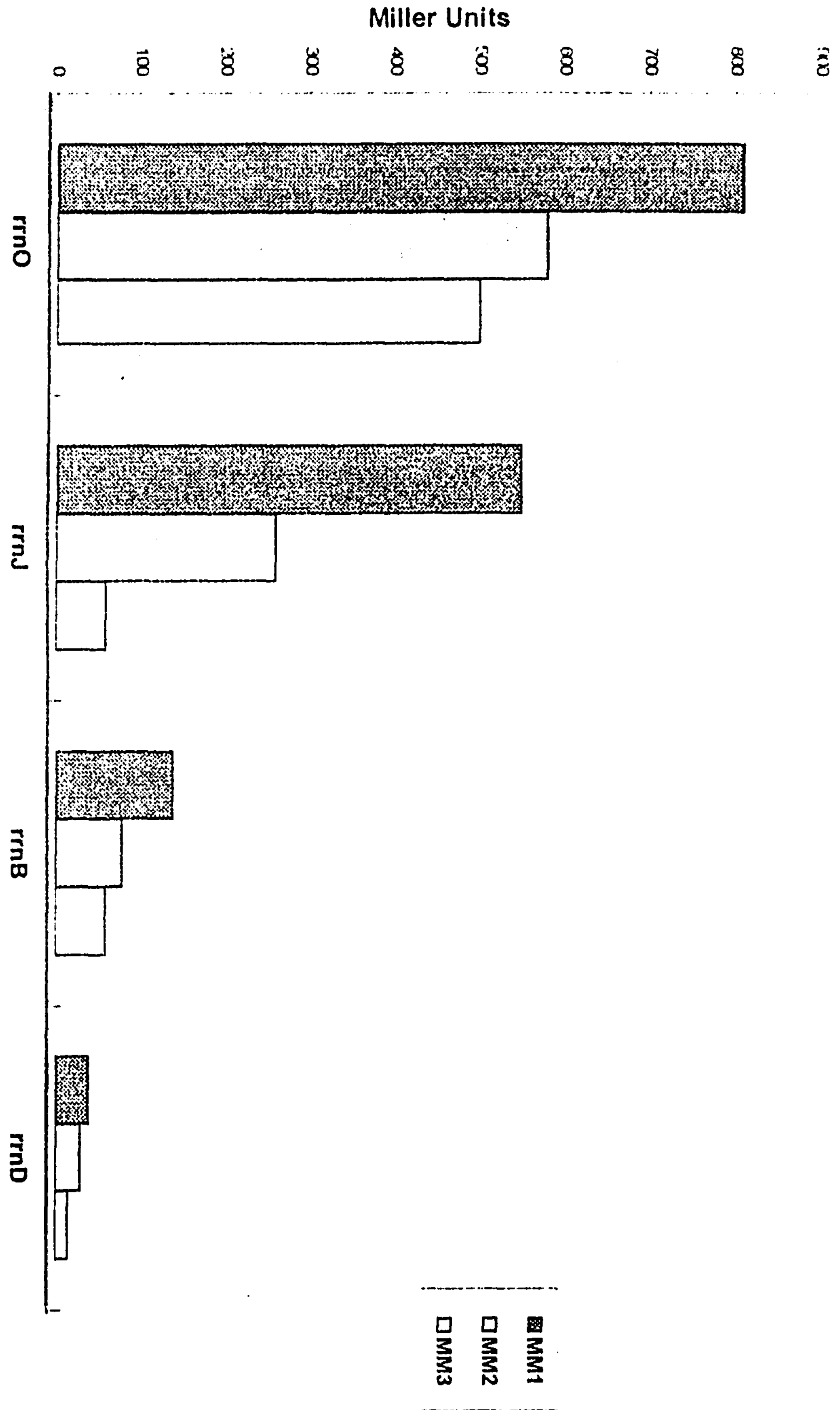
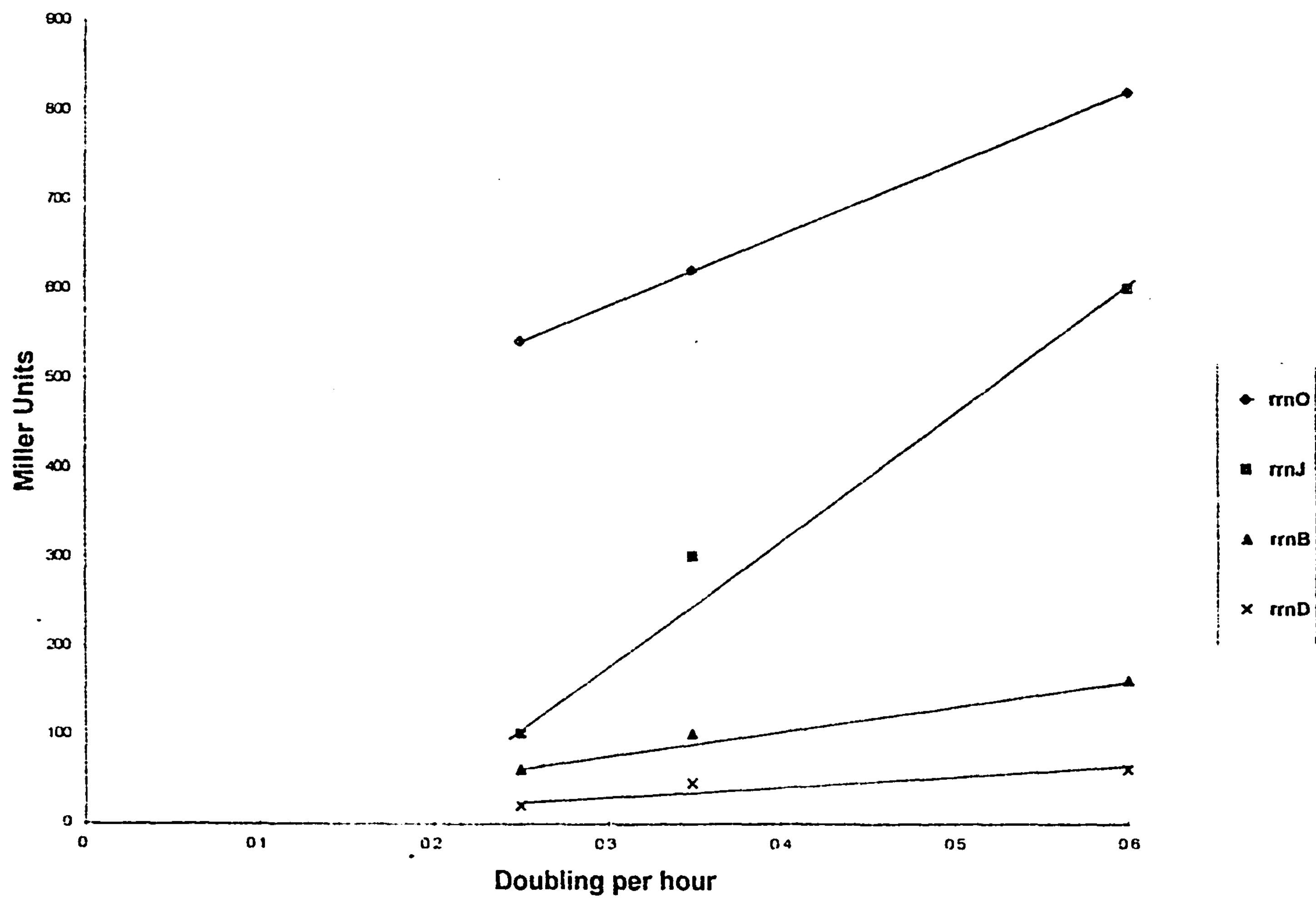


Fig. 26 The relative promoter strength of four rRNA operons as a function of growth rates. Various levels of β-galactosidase produced by *B. subtilis* strain IS58 containing integrated *lacZ* fusion: pPW4-*rrnO*, pWAR118-*rrnJ*, pAWR123-*rrnB*, and pAWR116-*rrnD*. One milliliter cell samples from cultures growing in MM1, MM2, and MM3 were taken at 100 Klett units and processed as described in materials and methods. The *rrn* activities were determined by Miller units.



Miller Units
0
100
200
300
400
500
600
700
800
rrnO
rrnJ
rrnB
rrnD
MM1
MM2
MM3

Fig. 27 Plot of β-galactosidase activity versus growth rate for *rrn* operons (*rrnO, rrnJ, rrnB, rrnD*) in *B. subtilis*. The slope indicates growth rate regulation. One milliliter cell samples from cultures growing in MM1, MM2, and MM3 were taken at varying Klett units (50, 100, 150) and processed as described in materials and methods. The β-galactosidase activity were determined by Miller units.



900
800
700
600
500
400
300
200
100
0
Miller Units
0
01
02
03
04
05
06
Doubling per hour
rrnO
rrnJ
rrnB
rrnD

Fig. 28 Expression levels of *rrnJ* operon as a function of growth rates. Various levels of β-galactosidase produced by *B. subtilis* strains IS58 and IS56 containing integrated *lacZ* fusion: pLR210, and pLR105 respectively. One milliliter cell samples from cultures growing in VY, MM1, MM2, and MM3 were taken at 80 Klett units and processed as described in materials and methods. The β-galactosidase activity were determined by Miller units.

Expression of *rrnJ* promoter parts P1 and P2 in different growth media

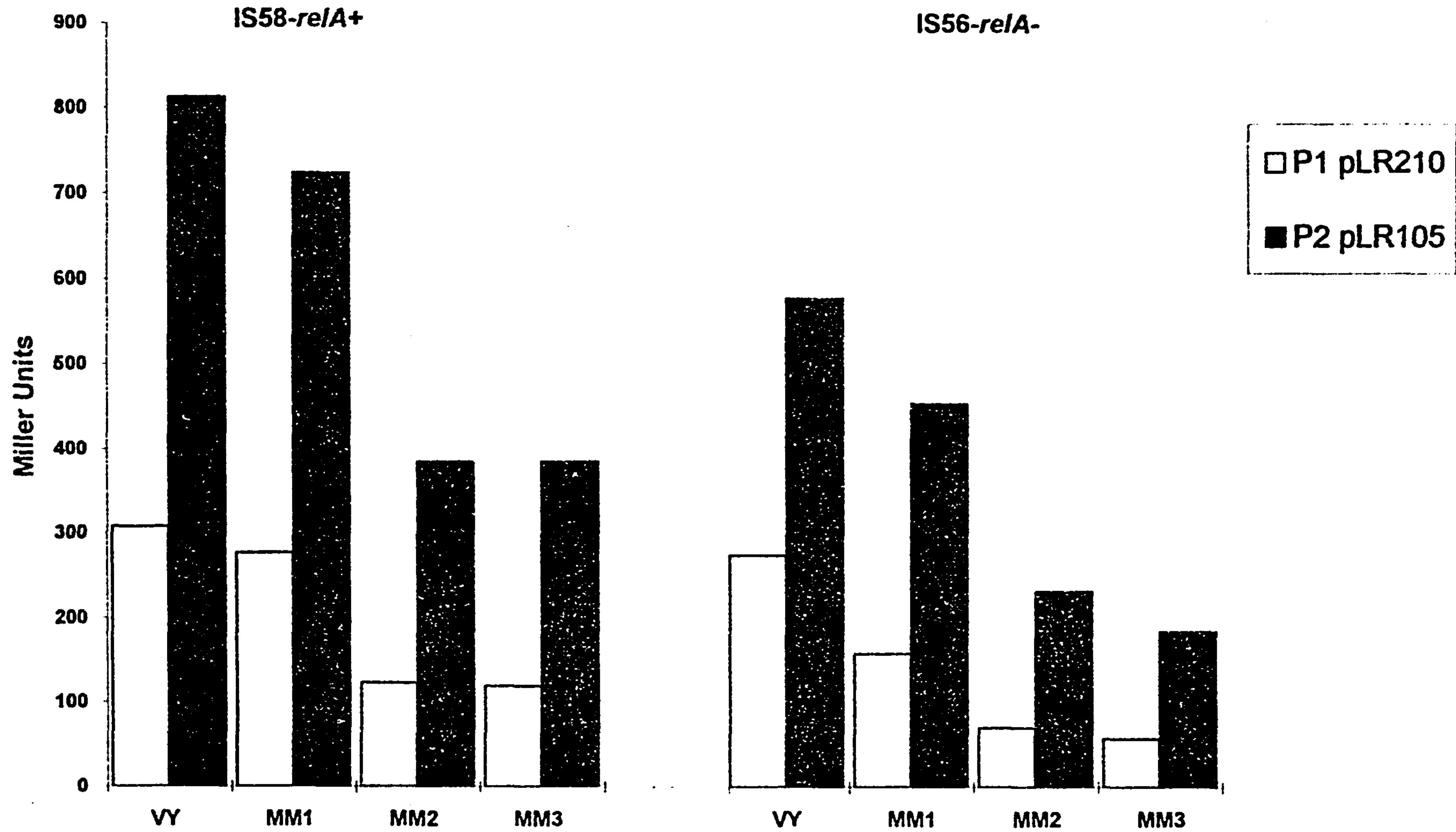


Fig 29 The expression of *rrnO* operon as a function of growth rates. β-galactosidase measurements were made form the three *B. subtilis* strains ISR58. ISR56 and LR3 containing integrated lacZ fusion pPW4-*rrnO* respectively. One milliliter cell samples from cultures growing in MM1, MM2, and MM3 were taken at 80 Klett reading and processed as described in materials and methods. The *rrn* activities were determined by Miller units.

Expression of *rrnO* promoter in three isogenic strains which are mutant in the *relA* and *rpoB* genes.

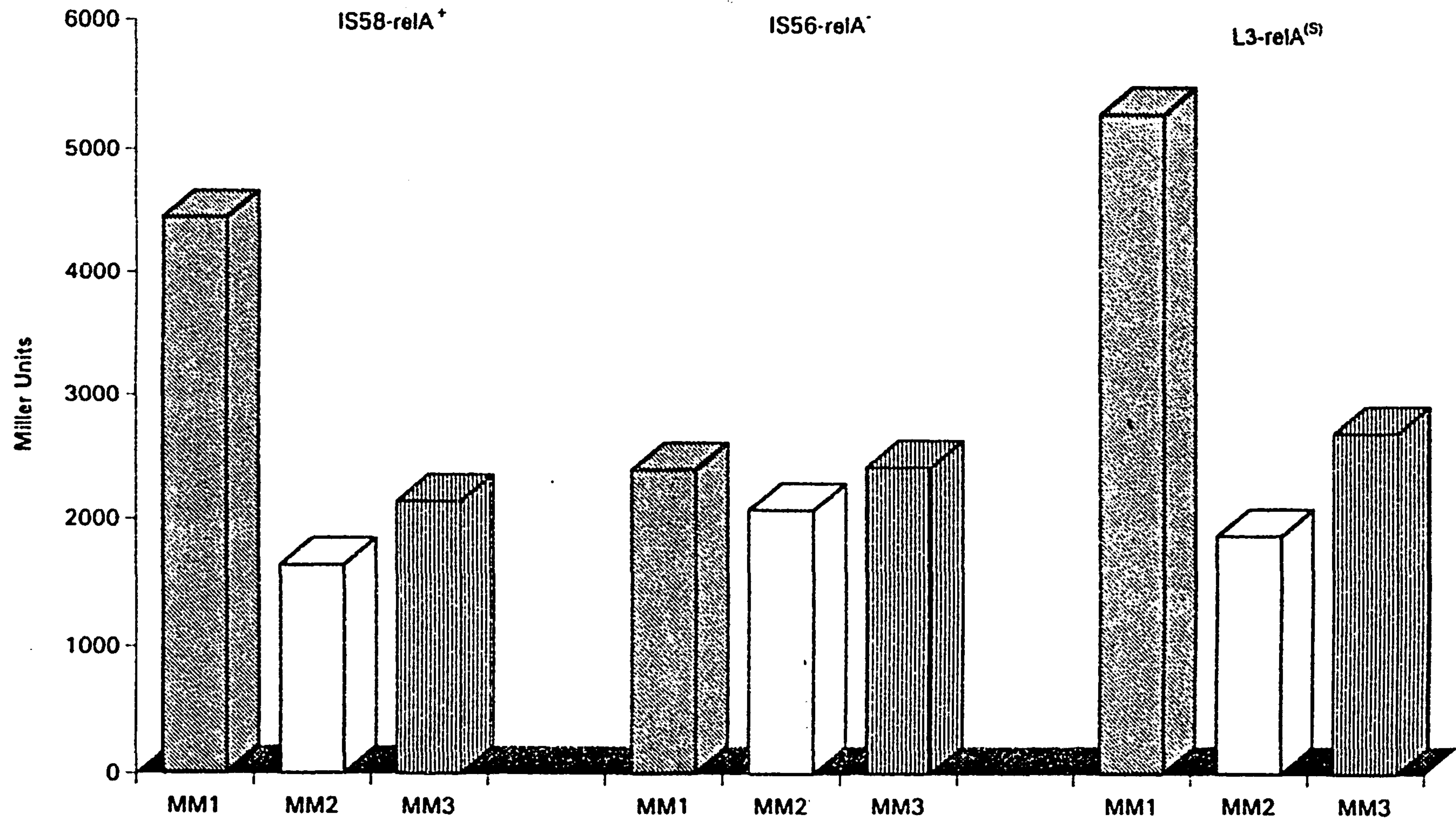


Fig. 30 The expression of *veg* gene (which is used as a control) as a function of growth rates. β-galactosidase measurements were made form the two *B. subtilis* strains ISR58 and ISR56 containing integrated lacZ fusion pPW810-*veg* respectively. One milliliter cell samples from cultures growing in MM1, MM2, and MM3 were taken at 80 Klett reading and processed as described in materials and methods. The *rrn* activities were determined by Miller units.

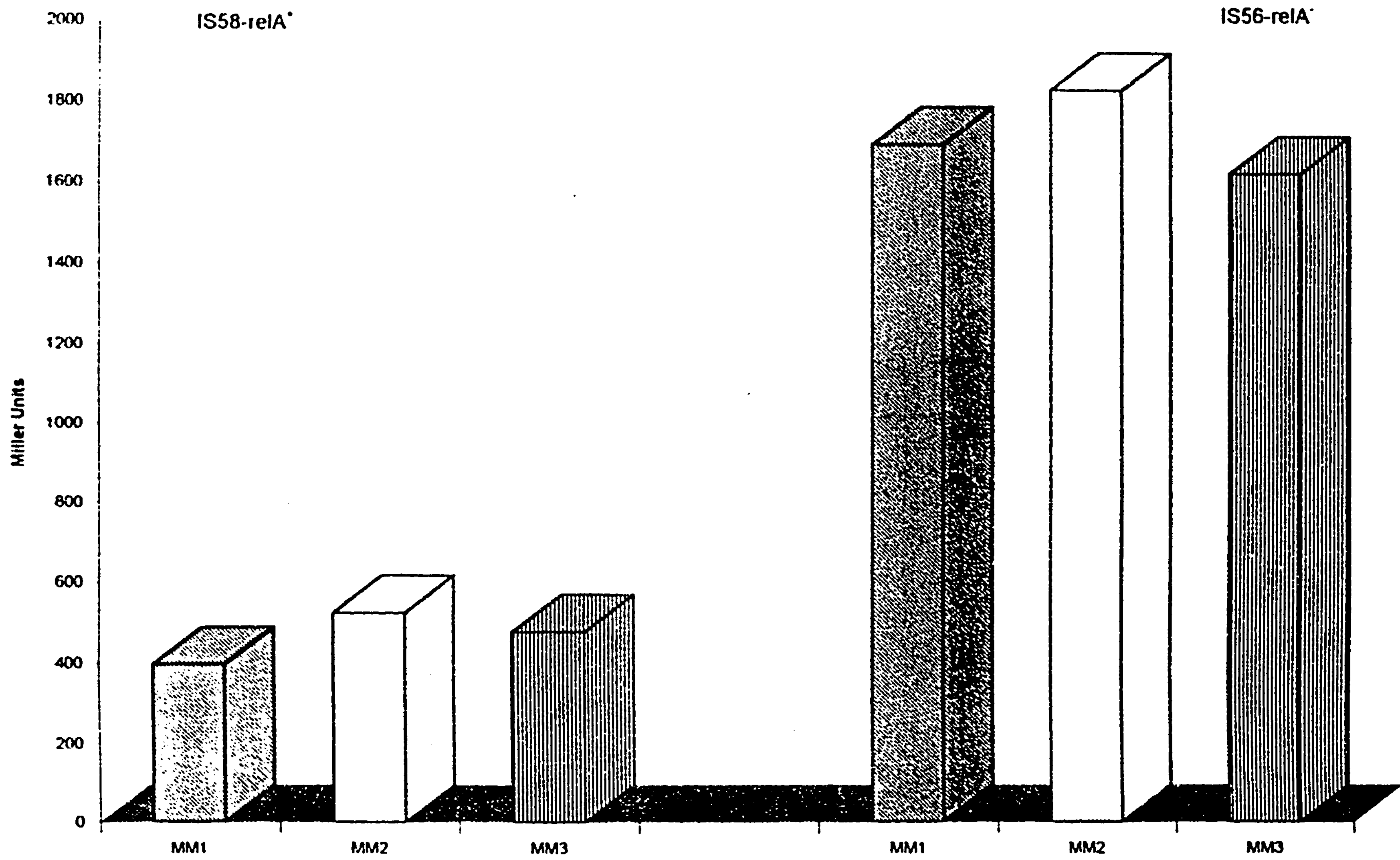

IS58-relA^{+}
IS56-relA^{-}
Miller Units
2000
1800
1600
1400
1200
1000
800
600
400
200
0
MM1
MM2
MM3
MM1
MM2
MM3

Fig. 31 The expression of *rrnO* operon as a function of growth rates as assayed by RNA dot blot. Cell cultures grown in MM1, MM2 and MM3 medium. RNA was isolated form the three *B. subtilis* strains which are rif^R ISR58, ISR56 and LR3 containing integrated *lacZ* fusion pPW4-*rrnO* respectively. 5 and 10 µg/ml RNA samples, were loaded onto nitrocellulose inserted in slot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determine by hybridization to probe specific for lacZ message. The probe was labelled with the random primer extension kit, using $[\alpha\text{-}^{32}P]$dCTP.

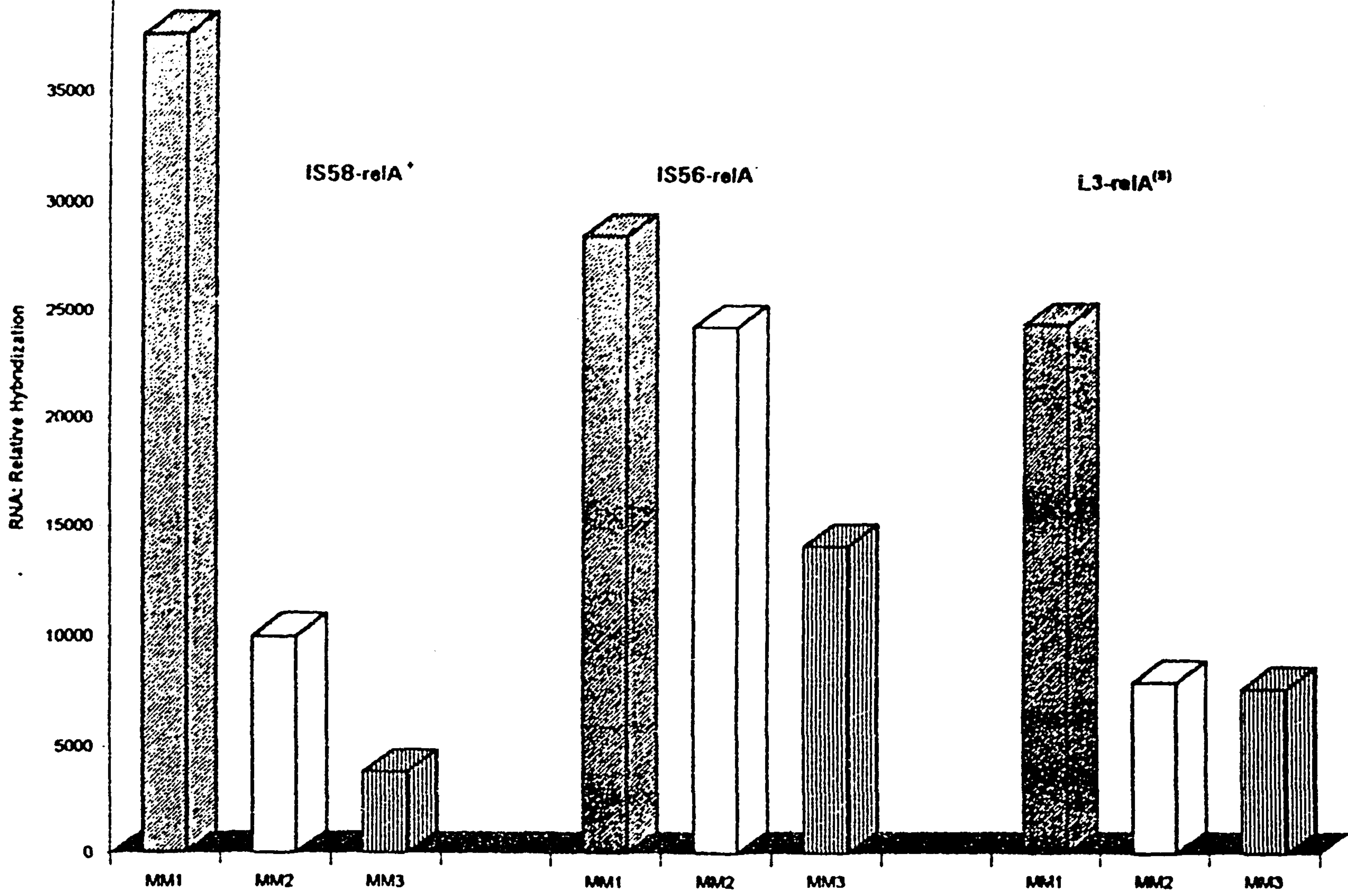
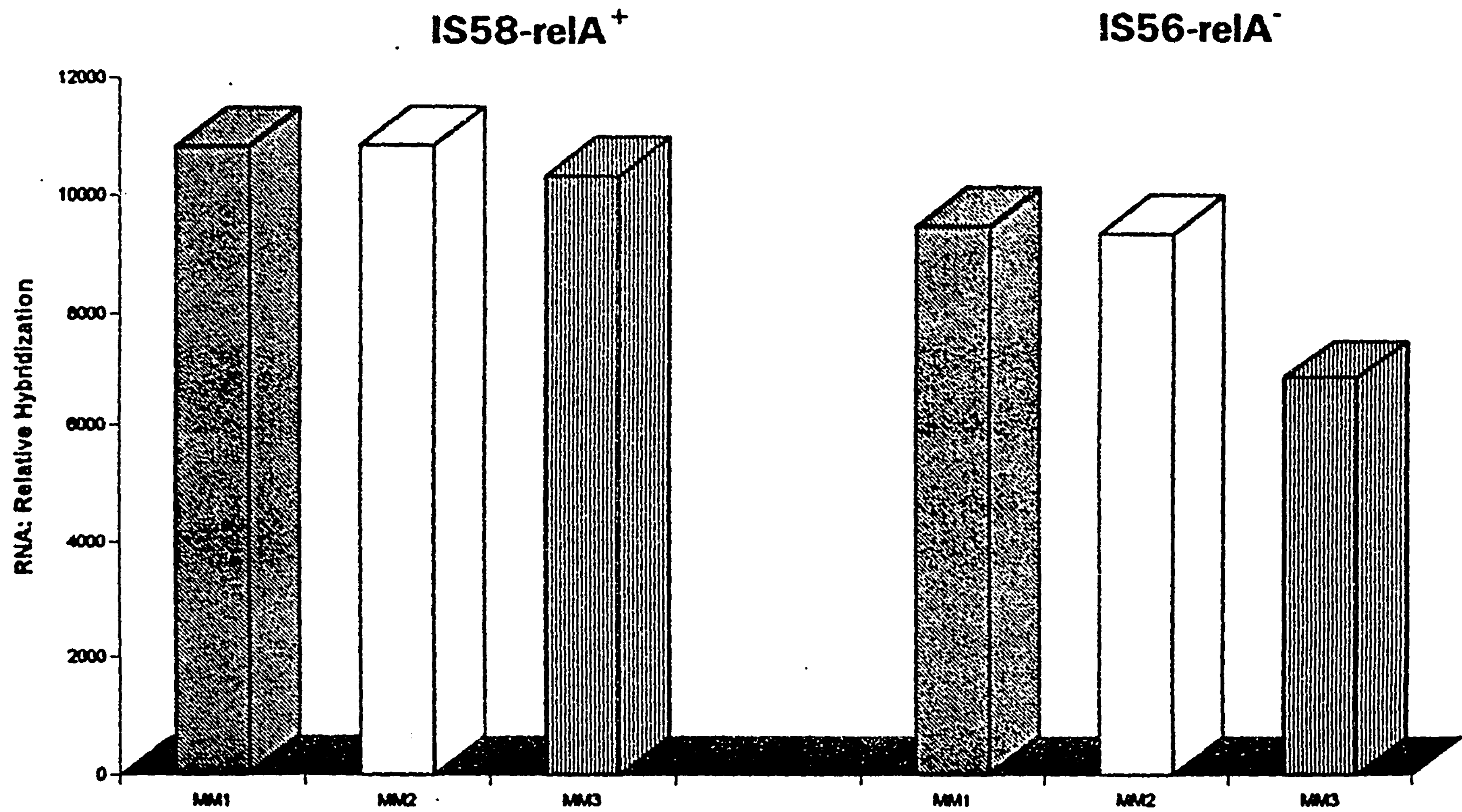

RNA: Relative Hybridization
0
5000
10000
15000
20000
25000
30000
35000
IS58-relA
IS56-relA
L3-relA
MM1
MM2
MM3
MM1
MM2
MM3
MM1
MM2
MM3

Fig. 32 The expression of *veg* operon (control) as a function of growth rates as assayed by RNA dot blot. Cell cultures grown in MM1, MM2 and MM3 medium. RNA was isolated form the two *B. subtilis* strains ISR58 and ISR56 containing integrated *lacZ* fusion pPW810-*veg* respectively. 5 and 10 µg/ml RNA samples, were loaded onto nitrocellulose inserted in dot blot apparatus. The filters were baked, prehybridized, hybridized and washed as described in (63). The levels of *lac-Z* mRNA in total RNA were determine by hybridization to probe specific for lacZ message. The prob was labelled with the random primer extension kit, using [α-^{32}P]dCTP.

Expression of *veg* promoter in two isogenic strains



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