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Widom, Russell Lee, Ph.D.

City University of New York, 1988

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HETEROGENEITY OF RIBOSOMAL RNA OPERONS IN BACILLUS SUBTILIS

by Russell Lee Widom

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.

1988

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ABSTRACT

HETEROGENEITY OF RIBOSOMAL RNA OPERONS IN BACILLUS SUBTILIS

by

Russell Lee Widom

Adviser: Rivka Rudner

The heterogeneity of rRNA operons in Bacillus subtilis was examined on three major levels. Heterogeneity among strains with respect to the number of rRNA gene sets was examined. Some strains of B. subtilis have only 9 instead of 10 gene sets, with specific deletion of either rrnG or rrnW. These deletions occur only where gene sets are found in tandem arrays, with rrnG being clustered next to rrnI and rrnH, and rrnW in a newly discovered tandem with rrnJ. Deletions of these gene sets can occur during plasmid insertion into the chromosome, or spontaneously. Rescue of chromosomal DNA from the site of such spontaneous deletions indicates that homologous recombination between tandem repeats is the most likely mechanism to generate strains lacking rRNA gene sets.

Heterogeneity at the DNA sequence level was studied by sequencing the DNA upstream of rrnJ and comparing it to previously sequenced regions. Analysis shows that B. subtilis rRNA leaders and promoters are heterogeneous whereas the analogous sequences in E. coli can be easily put into 2 highly homologous groups. The B. subtilis rrnJ

promoters cloned in E. coli exhibit growth-rate regulation, as expected for ribosomal promoters, indicating that the sequence requirements are conserved between the two organisms.

Heterogeneity of expression was examined by creating rrn-lacZ transcriptional fusions using an insertional plasmid with a promoterless lacZ gene. Measurements of beta-galactosidase activity indicate that the rrn gene sets can be grouped into strong (rrnO,rrnW), intermediate (rrnA,rrnE), and weak (rrnB,rrnD) levels of expression. The same fusions measured during spore formation shows a decrease in activity of all rrn-lacZ fusions, with a plateau at T₃ (3 hours after sporulation begins). The strong and intermediate operons mentioned above all reach a similar plateau, and the weak operons reach a similar, lower plateau.

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INTRODUCTION.

Ribosomal operons are unique among procaryotic genes not only because of their central role in metabolism but also because of their multiplicity, which gives rise to genetic variability. Ribosomal genes coding for 16S, 23S, and 5S rRNA species are organized into single transcriptional units in most bacteria; some have transfer RNA genes as part of the operon, in the 16S-23S intergenic spacer or at the 3' end of the 5S gene or both. Most of what is known about ribosomal genes comes from work done in Escherichia coli. Although many of the regulatory features will prove to be similar in Bacillus subtilis, some additional controls and the genomic arrangement of ribosomal genes are expected to differ because of the sporulation and germination functions which are part of the life cycle.

Multiplicity of Ribosomal Operons.

Most bacteria contain multiple copies of ribosomal gene sets. Halobacterium halobium, an archaebacteria, has been reported to contain one or two rRNA gene sets (Hofman et al., 1979, Neumann et al., 1983). Other slow growing bacteria seem to have small numbers of rRNA genes. Mycobacterium bovis BCG has one or two gene sets (Suzuki et al., 1987), as does Mycoplasma PG50 (Rasmussen et al., 1987,

Sawada et al., 1984). Other organisms with only two ribosomal gene sets include Thermus thermophilus HB8 (Ulbrich et al., 1984), Anacystis nidulans (Tomioka et al., 1981), Chlamydia trachomatis (Engel and Ganem, 1987), and Caulobacter crescentus (Feingold et al., 1985). The correlation between growth rate and operon number, proposed by Amikam et al., 1982, may not be absolute, as an archaebacteria, Methanococcus vanielii, contains four ribosomal gene sets (Jarsch et al., 1983), and a small number of operons does not prevent Caulobacter crescentus from undergoing a complex developmental process leading to formation of a sessile stalk cell from a motile swarmer cell (Ely and Shapiro, 1984).

The most studied organism, Escherichia coli, has been shown to have seven ribosomal operons (Kiss et al., 1977). The closest relative of this gram-negative enteric bacteria, Salmonella typhimurium, also contains seven operons, with nearly identical organization and chromosomal location, as demonstrated by intergeneric matings with E. coli K12 (Lehner et al., 1984). Streptomyces lividans TK21, a gram-positive bacterium, has at least 6 rRNA gene sets (Suzuki et al., 1988). The gram-positive bacterium Bacillus subtilis has been shown to have ten ribosomal gene sets (Stewart et al., 1982, Bott et al., 1984, LaFauci et al., 1986), the most seen among procaryotes that have been studied.

The ribosomal operon complement of particular

bacteria must afford some kind of selective advantage in order to maintain a given number of gene sets. It is apparent that deletions of ribosomal genes do occur but cases studied in E. coli and B. subtilis showed no effect of the deletion. Ellwood and Nomura, 1980, deleted a rRNA operon in E. coli, leaving the strain with only six. Measurements of growth rate in L-broth or minimal glucose medium revealed no difference from the control strain. RNA synthesis rates after nutritional shift-up were not affected by the deletion either. Deletions of rRNA genes have also been noted in B. subtilis (Loughney et al., 1983, Gottlieb et al., 1985, LaFauci et al., 1986, Widom et al., 1988). Strains containing nine rather than ten gene sets grow and sporulate the same as control strains (Loughney et al., 1983, LaFauci et al., 1986, Widom et al., 1988). Although not reported in B. subtilis, duplications of rRNA gene sets have been seen in both E. coli and S. typhimurium. Hill et al., 1977, reported tandem duplications in E. coli resulting from recombination between ribosomal genes; the same phenomenon occurred in S. typhimurium (Anderson and Roth, 1981). Although no physiological measurements were made, these duplications were unstable and required selection to be maintained.

Not only do alterations in the number of operons occur, but chromosomal rearrangements due to the presence of multiple rDNA sequences have been seen in E. coli and S.

typhimurium. The duplications of rRNA genes described above are accompanied by duplications of chromosomal segments found between two rRNA gene sets. Unequal crossing over between sister chromosomes can account for duplications of these regions. Anderson and Roth, 1981, demonstrated that one region of the S. typhimurium chromosome between two rRNA operons was duplicated in as many as 3% of cells in a culture. Other phenomenon have been seen in E. coli involving rRNA operons, including inversions between rRNA operons (Hill and Harnish, 1981) and translocations of regions bounded by rRNA genes (Hill and Harnish, 1982). Similar events occur in S. typhimurium (Lehner and Hill, 1980) but these events have not been associated with ribosomal operons in B. subtilis where far less has been investigated. One case has been described where a large chromosomal inversion has taken place just upstream of rrnD (E.D. Jarvis, unpublished results), but involvement of rDNA sequences has not been proven. The multiplicity of ribosomal RNA operons seems to be maintained even though chromosomal instability is the cost.

Ribosomal Operon Heterogeneity.

The seven ribosomal operons in E. coli are not identical with respect to their composition. All E. coli (and S. typhimurium) rRNA operons contain tRNA genes in the

16S-23S spacer region (Lehner et al., 1984, Lund et al., 1976) however two classes of spacer exist. Morgan et al., 1977, demonstrated that rrnB, C, E, and G have tRNA^{Glu} in their intergenic space, while rrnA, D, and H have tRNA^{Ile} and tRNA^{Ala}. In addition to these spacer tRNAs, several gene sets have tRNA genes at their 3' end, downstream of the 5S rRNA determinant (Morgan et al., 1978). Operon C has tRNA^{Asp} and tRNA^{Trp} as part of the operon; rrnH has a tRNA^{Asp} gene. Duester and Holmes, 1980, showed that downstream of rrnD is a tRNA^{Thr} gene followed by a second 5S gene.

In addition to these variations in the transcription unit, rrnA contains a variant 5S gene, called 5S III, in place of the normal 5S gene found in the other six operons (Morgan et al., 1977). These differences may be responsible for the maintenance of rRNA gene copy number, as deletions of specific gene sets may be prevented by coupling the rRNA genes to essential tRNA or 5S determinants.

Heterogeneity is also seen in Bacillus subtilis among the ten ribosomal gene sets. Only two of the ten operons contain any tRNA genes in the 16S-23S intergenic space (Stewart et al., 1982, Loughney et al., 1982, Ogasawara et al., 1983, LaFauci et al., 1986). Operon O and A have tRNA^{Ile} and tRNA^{Ala} determinants in the spacer region (Loughney et al., 1982, Ogasawara et al., 1983), similar to E. coli operons A, D, and H. Unlike E. coli, large clusters

of tRNA genes are found at the 3' end of several rRNA operons. A group of 21 tRNA genes is found at the 3' end of rrnB (Green and Vold, 1983, Wawrousek et al., 1984, Green et al., 1985). These genes are thought to be transcribed as part of the rrnB operon, but a possible promoter sequence is present after the first tRNA gene of the cluster (Vold, 1985, Vold and Green, 1986). A second smaller cluster of 16 tRNA genes are found at the 3' end of another rRNA operon as yet unidentified (Wawrousek et al., 1984). The final tRNA^{Leu} in this cluster seems to have its own promoter, but the preceeding 15 genes are part of the rRNA operon. Preliminary evidence from Rudner's lab indicates at least two tRNA genes exist at the 3' end of rrnE. A cluster of three ribosomal operons exists in B. subtilis where gene sets are separated by 1kbp. or less (Chow and Davidson, 1973, Stewart et al., 1982, Widom et al., 1988). In the spacer between operons I and H are found six tRNA genes, presumably part of the transcriptional unit of the upstream rRNA operon. Another cluster of 8 to 12 tRNA genes is predicted to be in the space between another tandem (Vold, 1985). The rrnH-rrnG tandem is only separated by 300bp. (Chow and Davidson, 1973, Widom et al., 1988), not large enough to contain 8 to 12 tRNA determinants, so this cluster should be found between rrnJ and rrnW, a newly discovered tandem (Widom et al., 1988, Jarvis et al., in press; see below).

As in E. coli, B. subtilis also has one rRNA operon which encodes a minor species of 5S rRNA (Raue and Planta, 1977); this is found just upstream of the cluster of 16 tRNA genes (Wawrousek et al., 1984). Other differences exist between the rRNA operons in B. subtilis. Ogasawara et al., 1983, noted heterogeneity in the 23S-5S spacer region. Their comparison of rrnA with rrnO revealed a 78bp. insert in the rrnA spacer. Examination of the same spacer which occurs between a 23S gene and the minor 5S species shows a 56bp. insert which is located 6bp. upstream of the position of the rrnA insert (Wawrousek et al., 1984). The significance of these differences is unknown, but they have not been reported in E. coli.

Less heterogeneity is seen in E. coli when one examines the upstream regulatory sequences. These sequences are responsible for the complex control of rRNA gene expression which includes stringent control, growth-rate regulation, feedback inhibition, and antitermination. DNA located upstream of all seven rRNA operons of E. coli has been sequenced (compiled in Jinks-Robertson and Nomura, 1987) and operons can be placed in two groups based on similarity of sequence in the few areas which show some variation. Operons A,B,C, and G have highly homologous sequences in these variable regions; operons D,E, and H make up the second group. All seven operons have a dual promoter structure, with the upstream promoter (P1) 100 base pairs in

front of the second promoter (P2) which lies 190 base pairs from the mature terminus of the 16S rRNA. Other regulatory sequences are found in the upstream region (see below) and are common to all seven operons, although all functional studies have not been done on all promoter regions.

In contrast to E. coli, Bacillus subtilis ribosomal operons have greater heterogeneity in the upstream regions. The sequence of at least six upstream regions has been determined (rrnO,A; Ogasawara et al., 1983, rrnB; Stewart and Bott, 1983, rrnH; P. Zuber PhD. dissertation, 1981, University of Wisconsin, Wawrousek and Hansen, 1983, rrnJ; this study, rrnD; E.D. Jarvis unpublished results) and several important differences exist. A 65bp. sequence is found immediately upstream of the mature 16S 5' terminus in rrnO, rrnB, and rrnD only. The significance of this sequence is not known, but one study (Ogasawara et al., 1983) used this fragment to successfully protect from S1 nuclease RNA isolated from germinating spores. The RNA was stable to rifampicin treatment, indicating that the RNA may play a role in germination. Variation exists in the spacing of the dual promoters, with 62bp. separating the P1 and P2 of rrnO and only 45bp. separating the rrnA tandem, with other operons having intermediate values. Operon H is the middle operon in the rrnI,H,G cluster and sequence analysis of its upstream region indicates only a single promoter exists in the 5' sequences, resembling P2 in its location

(P. Zuber, PhD. dissertation 1981, University of Wisconsin, Wawrousek and Hansen, 1983). The same is probably true for rrnG which is only 300bp. downstream of rrnH, and rrnW which is 1200bp. 3' to rrnJ. It remains to be determined whether these single promoters can perform all of the regulatory functions that are required; if not, ribosomal gene clusters must be regulated as a single unit. Finally, the P1 promoter of rrnO has only 16bp. separating the -35 and -10 consensus sequences. This could be important for the regulation of rrnO in particular (see below) as the normal 17bp. spacing is seen in all other rrn promoters thus sequenced.

Regulation of Ribosomal RNA Genes.

Regulation of the genes coding for rRNA has been studied extensively in E. coli. In order to support rapid growth, E. coli must synthesize many ribosomes, which are complex structures made up of 52 proteins and 3 rRNA species. The energy cost is great, so control of their production must be fine-tuned to the nutritional environment so as not to expend energy needlessly. Under certain conditions rRNA can account for 50% of total cellular transcripts (Gausling, 1977, Gausling, 1980, Bremer and Dennis, 1987), yet the operons account for only 0.5% of the genome (Jinks-Robertson and Nomura, 1987). This indicates

that the promoters are exceptionally strong as well as responsive to environmental changes. The DNA sequences required for regulation are those which are found in the upstream region, although regions within the operon have been suggested to play some role (see below and Li et al., 1984).

Growth-rate Regulation.

The number of ribosomes in a growing cell depends on the environment and increases with growth rate (Gausling, 1977, Jinks-Robertson and Nomura, 1987). Under most conditions, ribosome production is controlled so that all existing ribosomes are engaged in protein synthesis (Nomura et al., 1984). Experiments on isolated promoters of E. coli ribosomal operons have shown the relationship between growth rate and promoter activity. Miura et al., 1981, fused the rrnE P1P2 promoter pair to a lacZ gene and inserted this into a lambda phage genome which was then used to lysogenize E. coli strains. Two ribosomal protein operon promoters (spc and alpha) were also measured in the study. Enzymatic activity of beta-galactosidase was measured in cells growing at different rates. Beta-galactosidase activity increased with increasing growth rate only when the ribosomal promoters were used. This indicates that the ribosomal promoters are growth-rate regulated and that growth-rate

regulation of ribosomal proteins must take place at the translational level. In a similar but more complete study, Gourse et al., 1986, examined the rrnB promoters using the same expression system. They subcloned fragments of the promoter region and found that only P1 was growth-rate regulated; P2 gave a constant level of expression. The region responsible for this regulation was further narrowed down by creating fusion promoters, using the -35 region of P1 and -10 from the lac promoter (rrn-lac) or the opposite combination (lac-rrn). Only the promoters containing at least nucleotides -50 to -20 with respect to the P1 transcription start site were still growth-rate regulated. This shows that the -35 region of P1 is necessary for growth-rate regulation. More importantly, differential control of P1 and P2 is demonstrated, as was seen by Sarmientos and Cashel, 1983, who cloned the rrnA promoters on a plasmid and examined plasmid encoded transcripts. In their system, P1 transcripts increased with growth rate, not P2 transcripts.

An additional type of control may be used for rapid changes in nutritional conditions. Transcription of rRNA genes upon nutritional shift-up is rapidly but modestly increased within 2 minutes, as demonstrated by pulse-labeling of RNA and quantitation using rRNA specific probes (Zengel and Lindahl, 1986). The cells eventually reach transcription levels characteristic of the medium they are

growing in. Lukacsovich et al., 1987, noted the same phenomenon upon shift to rich medium using rrn-lacZ fusions and measuring beta-galactosidase activity. In their study, a rapid rise in enzyme activity accompanied shift to rich medium. This does not appear to be the same as normal growth-rate regulation because both the P1 and P2 promoters behave alike.

Feedback Inhibition.

The amount of rRNA synthesized is independent of the gene copy number. This phenomenon indicates some negative regulatory mechanism is at work. The amount of ^{32}P rRNA per unit cell mass is not altered when extra rRNA operons are introduced into cells on a multicopy plasmid (Ikemura and Nomura, 1977). Jinks-Robertson et al., 1983, introduced the rrnD operon in seven copies on a plasmid and measured rRNA synthesis rates. The rate of synthesis of rRNA did not change, indicating that despite doubling the number of operons, the total amount of rRNA remains constant. A negative feedback mechanism must depress each ribosomal promoter in order to maintain the same level of rRNA. If a plasmid with a large deletion in the rRNA operon is introduced to cells, no depression of chromosomal rRNA operons is seen, as judged by levels of co-transcribed tRNA genes, indicating that some product of the intact operon

(rRNA) is responsible for feedback regulation. A conditional expression system consisting of the rrnB operon fused to the lambda P_L promoter on a plasmid was used by Gourse et al., 1985, to examine rRNA synthesis after conditional expression of plasmid-borne rRNA operons. The fusion plasmid was introduced into a strain carrying a ts CI gene. Upon shift to 42°C, the P_L is activated, giving a two-fold increase in 30S and 50S ribosomal subunits, but not polysomes. Measurements of tRNA cotranscribed by chromosomal rRNA operons showed a 60% inhibition relative to controls with a deleted rrnB in the lambda P_L fusion. The region responsible for feedback inhibition was located by Gourse et al., 1986, by introducing an rrn operon on a multicopy plasmid into a cell lysogenized by a lambda phage with a rrn promoter-lacZ fusion. By measuring beta-galactosidase activity in cells containing lysogens with different upstream sequences, feedback control was seen only when the -35 region of P_L was still present. This is the same region required for growth-rate regulation, indicating that these two phenomenon might be identical. Feedback regulation may be caused by free non-translating ribosomes; at high growth rates there are few free ribosomes so no repression of rRNA synthesis results. When growth slows, fewer ribosomes are actively translating messages so they are available to repress further rRNA transcription. This mechanism, espoused by Nomura et al., 1984, may not explain

all of the observations, especially the involvement of guanine nucleotides (see below).

Stringent Control.

When cells are starved for amino acids, rapid changes occur, including a dramatic decrease in stable RNA production. This type of regulation, known as stringent control, has been correlated with the appearance in the cell of guanosine tetraphosphate (ppGpp; Cashel and Gallant, 1969). Mutant strains (relA⁻) show no drop in stable RNA synthesis under starvation conditions, but also show no increase in ppGpp levels (Gallant, 1979), therefore the inverse correlation between ppGpp levels and stable RNA gene activity is valid in normal and relaxed strains (Ryals et al., 1982). Guanosine tetraphosphate is thought to work at the level of RNA polymerase, causing a preferential decrease of polymerase binding to stringent promoters. A so-called stringent discriminator sequence has been described (Travers, 1980) which is a GC rich region downstream of the -10 box of stringently controlled promoters. This may not be sufficient to identify promoters which are subject to stringent control, as some non-stringent promoters have GC rich regions as well. Yamagishi et al., 1987, used a hybrid rRNA operon, with rrnB fused to lambda P_L (without the stringent discriminator) to measure the stringent response.

A normal stringent response was seen in relA⁺ but not relA⁻ cells, indicating that the discriminator was not necessary. They also showed that many T7 phage promoters, which are strong, also exhibit stringent control, indicating that perhaps stringent control generally affects strong promoters, with no sequence requirements.

In-vitro studies using stringent and non-stringent promoters show a preferential effect on stringent promoters when ppGpp is added. Glaser et al., 1983, used the dual promoters of rrnA and looked at in-vitro transcripts under a variety of conditions. The addition of ppGpp caused inhibition of transcripts only from P1, as is seen in vivo. Kajitani and Ishihama, 1984, had the same observation with the separately cloned promoters of rrnE. They looked at other stringent and non-stringent promoters and found a correlation between the in-vivo and in-vitro response to ppGpp.

Sarmientos et al., 1983, and Gourse et al., 1983, used plasmid carried rrn operon promoter regions and deletion derivatives to measure plasmid directed transcripts during starvation. Both studies showed that rrn promoters on plasmids were subject to stringent control, but only the P1 promoter was affected. P2 does not decrease its activity upon starvation, and no sequences downstream of +20 from the P1 transcription initiation site are necessary for proper regulation, including sites at which RNA polymerase pauses

during in-vitro transcription of the region (Kingston and Chamberlin, 1981), once thought to play a role in stringent control.

Antitermination.

Another function specified by the leader region is antitermination. RNA polymerase which is transcribing a ribosomal operon must not terminate before reaching the end of the structural genes. Since this transcript is large (>5kb.) and is not translated, some mechanism must exist to prevent rho dependent termination at sites within the rRNA structural genes (Aksoy et al., 1984, Li et al., 1984). In protein coding regions, premature termination is prevented by concurrent translation of the message, but rRNA is not translated, so another mechanism must exist. Squires and co-workers have noted DNA sequences in the leader region of E. coli rRNA operons which resemble the nut antitermination sites of lambda phage. These sequences are found between the P2 promoter and the mature 16S terminus, from 170 to 100 base pairs upstream of the latter. If this region is inserted in a plasmid between a strong promoter and a rho dependent terminator, expression of a distal chloramphenicol acetyltransferase (CAT) gene is 50% of controls with no terminator as opposed to 1-2% with no antiterminator (Li et al., 1984). The effect is orientation dependent and is

abolished by translation through the antiterminator region, and does not depend on the transcription being promoted by ribosomal promoters (Li et al., 1984). Similar sequences can be found in the 16S-23S intergenic spacer and a similar role can be proposed.

Nomura and co-workers have demonstrated that antitermination in ribosomal operons is nusB dependent (Sharrock et al., 1985). NusB mutants of E. coli show an increase in 16S transcription relative to 23S, indicating that fewer full length transcripts are being made. Very little evidence supports the involvement of other nus proteins in rRNA antitermination. The nusA1, nusC60, and nusE71 mutations did not show the same effect, although one cold sensitive mutant (nusAcs) did show a decrease in antitermination at the non-permissive temperature (Sharrock et al., 1985).

The antitermination mechanism of rRNA operons is important to the overall regulation of macromolecular synthesis because premature termination leads to an imbalance of 16S and 23S rRNAs. Transcripts terminating before the 23S gene can give rise to a 16S RNA but not a 23S or 5S species. Feedback regulation will cause all ribosomal operons to be transcribed at a higher rate so that enough complete ribosomes can be synthesized to support a particular growth rate. Since tRNA genes are subject to feedback regulation, their levels also increase under such

conditions. The nusB5 mutant of E. coli shows an increase in rRNA and tRNA transcription rates, presumably stable RNA transcription is increased to compensate for the decrease in full length transcripts caused by the nusB5 mutation (Sharrock et al., 1985).

An additional feature of the rRNA leader is a terminator structure which is related to the antitermination mechanism. A structure is found between the antitermination sequences and the 16S gene, called t_L , which causes nusA but not rho dependent termination in vitro (Kingston and Chamberlin, 1981). If this region is deleted from the rrnB operon contained on a multicopy plasmid, the cells carrying the plasmid exhibit lower growth rates than controls. These cells showed premature termination of plasmid borne operon transcription and a compensatory increase in rRNA and tRNA transcription rates (Zacharias and Wagner, 1987). This terminator is proposed to function by causing any RNA polymerase which has not been modified at the antitermination sequences to terminate its transcription before it reaches the structural genes. This would prevent transcription termination from occurring within the structural region which might lead to an imbalance of 16S over 23S species. Although an imbalance of rRNA might occur, Siehnel and Morgan, 1985, have shown that excess 16S rRNA in cells is rapidly degraded and does not affect transcription of r-protein operons which are normally

repressed by r-proteins which bind to 16S rRNA.

Other Regulatory Features.

Several studies indicate that P1 is a strong promoter, responsible for most of the regulatory phenomenon (see above), whereas P2 is a low level maintenance promoter. Glaser et al., 1983, observed that in vitro, P1 was very active at a high RNA polymerase/DNA ratio, whereas at a low ratio, P2 was more active than P1. This indicates that RNA polymerase has a low affinity for P1 and a higher affinity for P2. The strength of P1 must be due to other factors which allow greater levels of transcription than P2. An AT rich sequence upstream of P1 is a candidate for this "upstream activator". Removal of this sequence in vitro does nothing to the expression of P1 (Petho et al., 1986) under standard conditions, but under more physiological conditions, P1 transcripts are reduced. Gourse et al., 1986, showed that when this region is present, expression of a fused lacZ gene is 15-fold higher than constructs lacking the region.

Bacillus subtilis rRNA Regulation.

Compared to E. coli, relatively few studies on rRNA control have been done in Bacillus subtilis. S1 nuclease

studies of in-vivo transcripts indicate that the P1 P2 promoter pair may not be used identically by all operons. Ogasawara et al., 1983, showed that P2 of rrnO was the major promoter for that operon, in contrast to rrnA, which showed more transcripts from P1. In vitro transcription showed that both E. coli and B. subtilis RNA polymerases favored P2 over P1 (Seiki et al., 1982) for rrnO. This may be due to the structural peculiarity of rrnO P1, having only 16bp. separating the -35 from the -10 region. Stewart and Bott, 1983, showed more transcripts from P1 of rrnB than from P2, but this may be due to the very short (29bp.) fragment of RNA protected from the P2 promoter. Interestingly, the promoters of rrnB, when cloned in front of a CAT gene on an E. coli plasmid, are growth-rate regulated, suggesting that the regulatory sequences are conserved between organisms (Deneer and Spiegelman, 1987). Although growth-rate regulation is seen, the promoter specificity is opposite from that of E. coli. The B. subtilis rrnB P2 promoter is growth-rate regulated.

Stringent control is evident in B. subtilis and can also be correlated with cellular ppGpp levels (Swanton and Edlin, 1972, Nishino et al., 1979). One study indicates that ppGpp and pppGpp levels can be influenced by using transcription inhibitors such as rifampin or lipiarmycin (Price and Brown, 1981). The accumulation of guanosine polyphosphates in this case is independent of rel mutations,

suggesting a different mechanism of ppGpp production than in the normal stringent response.

Since Bacillus subtilis can sporulate, special regulation of rRNA may be necessary once the stationary phase of growth begins. Many transcriptional changes occur upon entry into sporulation, mediated by many regulatory proteins, including new RNA polymerase sigma factors, which change polymerase promoter affinities. The effects on rRNA synthesis have not been widely studied. Upon entry into stationary phase, rRNA production is reduced, so that at T_1 (one hour past the beginning of stationary) only 28% of total RNA synthesized is rRNA, as opposed to 65% during vegetative growth (Testa and Rudner, 1975). The amount of rRNA synthesized during sporulation increases until the fraction of transcripts which are rRNA is equal to that of vegetative growth. This may represent replacement of ribosomes made during vegetative growth so that translational machinery will be able to make proteins necessary for spore production. In order to investigate further the relationship between sporulation and rRNA synthesis, more systematic studies are needed, which may uncover elements of rRNA control that do not exist in the gram-negative enterics. The heterogeneity seen in rRNA operons of B. subtilis may also reveal a differential use of individual operons, which has not been seen in other procaryotic systems.

PURPOSES OF THIS STUDY.

- 1) To examine the heterogeneity seen in Bacillus subtilis with respect to the number and organization of rRNA genes and to explore the mechanisms which could lead to this heterogeneity.
- 2) To examine individual rRNA operon promoter regions in order to determine if any sequence heterogeneities can be related to functional heterogeneity.
- 3) To examine systematically the expression of ribosomal operons with the purpose of defining special roles for individual rRNA gene sets with respect to vegetative growth, sporulation, and germination.

MATERIALS AND METHODS

Strains and Plasmids Used.

The Bacillus subtilis and Escherichia coli strains used are listed in Table 1. Integrant strains are those transformed by insertional plasmids; the site of integration is specified. The plasmids used are listed in Table 2. The first group of plasmids were constructed previously, the second group were constructed for this study, and the third group were rescued in vivo from chromosomal DNA as described below.

Growth Media and Selective Plates.

Escherichia coli strains for competent cells or plasmid isolation were grown in LB broth (LB is 1% tryptone [Difco Laboratories, Detroit, MI.], 0.5% yeast extract [Difco], 0.5% NaCl, 0.1% glucose pH 7.0). When appropriate, antibiotics were added to LB after autoclaving at the following concentrations: ampicillin (Ap, Sigma Chemical Co., St. Louis, MO.), 100 ug/ml; tetracycline (Tc, Sigma), 15 ug/ml; chloramphenicol (Cm, Sigma), 10 or 50 ug/ml. For selective plates, 1.5% agar (Difco) was added to LB and the appropriate antibiotic added after autoclaving at the concentrations given above. E. coli minimal medium was M9

salts (Maniatis et al., 1972) (M9 is per liter: 6g Na_2HPO_4 , 3g KH_2PO_4 , 0.5g NaCl , 1g NH_4Cl with 0.1ml of 1M MgSO_4 and 0.1 ml of 0.1M CaCl_2 added separately after autoclaving) with 0.4% glucose as carbon source. M9 salts were supplemented with thiamine (2ml of a 1mg/ml solution) and leucine (1ml of a 10mg/ml solution). Cells for chloramphenicol acetyltransferase (CAT) measurements were grown in A media (Miller, 1972) (per liter: 10.5g K_2HPO_4 , 4.5g KH_2PO_4 , 1g $(\text{NH}_4)_2\text{SO}_4$, 0.5g Na citrate with 0.1 ml of 1M MgSO_4 added after autoclaving) supplemented with 2ml of 1mg/ml thiamine, 1ml of 10mg/ml proline and leucine, 50ug/ml Cm, and varying amounts of glucose (0.4 to 0.01%), yeast extract (0.4 or 0.01%), or 0.5% succinate in place of glucose to alter growth rates. Growth rates were determined from Klett readings by plotting log of Klett vs. time as described by Miller, 1972. Growth of JM101 for M13 bacteriophage infection was in 2xYT broth (YT is 8g tryptone, 8g yeast extract, and 5g NaCl per liter). Thick plates for plaque formation were YT plus 1.5% agar; top agar was YT plus 0.6% agar.

Bacillus subtilis strains were grown in VY medium (25g veal infusion broth [Difco] plus 5g yeast extract per liter) supplemented with 10ug/ml Cm when appropriate. Selection of Cm resistant cells was on LB plates with 10ug/ml Cm. Lincomycin and spectinomycin resistance was scored on LB plates with 50ug lincomycin or 125ug

spectinomycin per ml. GlpT⁺ recombinants are sensitive to 40ug fosfomycin per ml added to a low phosphate medium (Miki et al., 1965). Minimal medium was Spizizen minimal salts (Anagnostopoulos and Spizizen, 1961) (per liter: 6g KH₂PO₄, 14g K₂HPO₄, 2g (NH₄)₂SO₄, 1g Na citrate, 0.2g MgSO₄) supplemented with 100ug/ml of required amino acids, 1% Na glutamate, and 0.05% glucose. Minimal agar was prepared by mixing after autoclaving equal volumes of 2x Spizizen salts with 2% glutamate and 3% agar with 1% glucose and then adding appropriate amino acids to a final concentration of 100ug/ml. Strains were maintained as streaks on tryptose blood agar base (TBAB, Difco) plates supplemented with 0.2ml of 0.1M MnCl₂ and 5ml of 1M MgSO₄ per liter, and 10ug Cm/ml when appropriate. Amy⁺ cells were detected on TBAB plates with 1.0% potato starch. After overnight incubation, plates were flooded with 2ml of 0.5% (wt/vol) I₂ and 5.0% (wt/vol) KI (Nicholson and Chambliss, 1985), and Amy⁺ colonies exhibited an unstained halo of starch hydrolysis. Cells were sporulated in SG medium (per liter: 16g nutrient broth, 0.5g MgSO₄, 2g KCl, 1mM Ca(NO₃)₂, 0.1mM MnCl₂, 1uM FeSO₄, 1g glucose; Shaeffer et al., 1965). Viable plating was done on nutrient agar (Difco) plates.

Preparation of Competent Cells.

E. coli was made competent for plasmid uptake by CaCl₂

treatment of a mid-log phase culture (Mandel and Higa, 1970). Cells grown in LB broth to a klett of 80-100 (Klett-Summerson colorimeter, red filter), were chilled on ice for 10 minutes and pelleted at 5000 rpm for 5 minutes. Cells were resuspended in 0.5 volumes of sterile 0.01M CaCl_2 . Cells were pelleted again and gently resuspended in the same volume of 0.03M CaCl_2 and left on ice for 20 minutes. Competent cells were then pelleted and gently resuspended in 0.1 volumes of 0.03M CaCl_2 in 40% glycerol. Aliquots were placed in sterile plastic tubes and quick frozen in dry ice-ethanol. Frozen cells were stored at -70°C until needed. For a transformation assay, frozen cells were thawed out under cold running water and 0.2ml mixed with transforming DNA and kept on ice for 30 minutes. The mixture was heat shocked at 42°C for 2 minutes and 1.5 ml LB was then added before incubating the cells at 37°C on a culture roller for a minimum of 90 minutes. Cells were then plated on selective medium with appropriate dilutions made with sterile dilution salts (0.15M NaCl, 0.02M K_2HPO_4 pH 7.0).

B. subtilis was made competent by the method of Anagnostopoulos and Spizizen, 1961, as modified by Rudner et al., 1967. An overnight, 10ml VY culture was pelleted in a clinical centrifuge and resuspended in 2.5ml of Spizizen minimal medium I (1x Spizizen salts, 0.5% glucose, 0.02% casamino acids [Difco], 0.1% yeast extract, 0.8% L-arginine, 100ug/ml of required amino acids). This culture was used to

inoculate a sidearm flask containing 20ml 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 in Spizizen minimal medium II (1x Spizizen salts, 0.5% glucose, 0.01% casamino acids, 0.05% yeast extract, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05mM spermine tetrahydrochloride, 5ug/ml of required amino acids) and grown for 90 minutes, at which time the culture was used in a transformation assay or frozen for storage. Freezing was done by pelleting cells at room temperature in sterile centrifuge bottles, resuspending in 1/10th volume of minimal medium II with 5% glycerol, and quick freezing 2ml aliquots in a dry ice-ethanol bath. Frozen cells were reconstituted with minimal medium II after quick thawing at 65°C. Plasmid DNA (5-10ug) or chromosomal DNA (0.2 to 2 ug) in 0.1 ml was mixed with 0.9ml of fresh or reconstituted competent cells and was incubated on a culture roller for 30 minutes at 37°C. DNAase I (Cooper Biomedical) was added (0.1 ml of a 100ug/ml solution in 0.25M $MgSO_4$) and tubes were incubated an additional 10 minutes after which cells were plated with appropriate dilution onto LB plates with Cm or selective minimal plates. Plates were incubated at 37°C for 24 to 48 hrs.

PBS1 Phage Transduction.

Overnight cultures of B. subtilis in VY were used to

inoculate 10ml VY cultures (0.2ml into 10ml) and were grown to a Klett of 100 from a starting value of 15 at which time 0.2ml of PBS1 (109-1010 PFU/ml) was added. Incubation until Klett readings dropped was followed by the addition of DNAase I and incubation overnight without shaking. Lysates were centrifuged and filtered through 0.45 um nitrocellulose filters (Uni-flo syringe filter, Schleicher and Schuell [S+S], Keene, NH.). Mapping recipient strains were grown to a Klett of 300 and 1ml mixed with 0.1ml of transducing lysate, followed by incubation for 30 minutes at 37°C on a culture roller. Cells were plated on LB plates with Cm or on appropriate minimal plates. Linkages were tested by replica plating with sterile velvet.

DNA Isolation.

Plasmid isolation from E. coli began by inoculation of 500ml LB with 12-13ml of an overnight LB culture (starting Klett 20-25). All media was supplemented with appropriate antibiotics to maintain the plasmid. After 4 hrs. growth at 37°C with shaking (Klett =180), solid Cm was added to a final concentration of 180ug/ml followed by overnight incubation to amplify colEI replicons (Clewell and Helinski, 1972). Cells were chilled, pelleted, and plasmid DNA extracted by the method of Tanaka and Weisblum, 1975. Cell pellets were resuspended in 8ml of 50mM Tris-HCl pH

8.0, 25% sucrose and incubated on ice with 7mg lysozyme (Sigma) and 20ug/ml RNAase A (Cooper Biomedical) for 5 minutes. 0.8ml of 250mM EDTA pH 8.0 and 8ml of 0.3% Triton X-100 (Sigma), 188mM EDTA, 150mM Tris-HCl pH 8.0 was added and the suspension mixed gently and left on ice for 30 minutes. Cell debris and chromosomal DNA was pelleted by centrifuging for 90 to 120 minutes at 17,000 rpm. The supernatant was deproteinized by shaking with an equal volume of water and buffered phenol (Gallard Schlesinger, Carle Place, N.Y.; 80% phenol buffered to pH 8.0 with 2M tris base with 0.1% 8-hydroxyquinoline added as a preservative), followed by centrifugation at 10,000 rpm for 10 minutes at 40C. The aqueous phase was allowed to come to room temperature, Na acetate was added to 0.3M, and plasmid was precipitated with 3 volumes of 95% ethanol. After overnight precipitation at -200C, the plasmid was pelleted at 8000 rpm for 10 minutes. Two washes with 10ml of 80% ethanol preceeded drying the DNA under vacuum and final resuspension in 3ml of 10mM Tris-HCl pH 8.0, 50uM EDTA (TE). A typical recovery was 2-3 mg of plasmid DNA from a 500ml culture. Plasmids were stored at 40C in 5ml plastic snap-cap culture tubes (Falcon).

Bacillus subtilis chromosomal DNA was prepared by pelleting an overnight VY culture (2x 500ml), resuspending in 0.15M NaCl. 0.1M EDTA pH 8.0 (4ml per gram wet weight of cells), and incubation with lysozyme (7-10mg) at 370C for 1-

1.5 hrs. Sodium dodecyl sulfate was added to a 2.5% concentration and the lysate heated at 60°C for 10 minutes. DNA was extracted by the procedure of Marmur, 1961, as modified by Rudner et al., 1967. Concentration of purified DNA was estimated by its absorbance at 260 nm. Typically, 1mg of DNA was recovered per gram wet weight of cells. Stock DNA was diluted to 100ug/ml with TE and used as a working solution.

Rapid plasmid and M13 RF DNA isolation was by alkaline lysis (Birnboim and Doly, 1979). LB or 2x YT 5ml cultures of plasmid bearing or phage infected E. coli cells were pelleted in a tabletop centrifuge for 10 min., resuspended in 1.5ml of dilution salts, and then transferred to 1.5ml polypropylene tubes. Cells were pelleted in a microcentrifuge for 30 seconds and then resuspended in 200ul of solution I (50mM glucose, 10mM EDTA, 25mM Tris-HCl pH 8.0, 2mg/ml lysozyme, 250ug/ml RNAase A) and kept on ice for 30 minutes. 400 ul of solution II (0.2N NaOH, 1% SDS made fresh) was added and tubes were mixed by inversion and incubated 5 minutes on ice. 300ul of 3M Na acetate pH 4.8 was added and tubes were incubated for 60 minutes on ice after mixing. The protein and chromosomal DNA precipitate was removed by spinning in a cold microfuge for 15 minutes and decanting the supernatant into a new 1.5ml tube. Plasmid or M13 phage RF DNA was precipitated by adding an equal volume of isopropanol, mixing well by inversion, and

centrifuging for 10 minutes at room temperature. The isopropanol was removed and the DNA pellet washed once with 70% ethanol at room temperature. After removal of alcohol, the tubes were dried under a vacuum for 30 minutes and the pellets were resuspended in 50-200ul of water and heat treated at 68°C for 10 min. to inactivate any nucleases. DNAs were stored at -20°C until needed.

Single stranded M13 phage DNA isolation began by spinning down a 5ml culture of infected JM101 in 2x YT broth in a tabletop centrifuge. The pellet was used to isolate double stranded RF DNA. The supernatant was transferred to a new tube and spun a second time and decanted into a clean 16x120mm culture tube. Phage was precipitated by adding 1ml of 12.5% polyethylene glycol 6000 (PEG, Eastman Kodak, Rochester, NY.), 2.5M NaCl to 5ml of supernatant. After extensive vortexing, the tubes were kept at room temperature for 10 min. and then centrifuged 10 min. in the tabletop centrifuge. The supernatant was discarded and a water aspirator used to remove any traces of PEG followed by drying the inside of the tube with a lint-free towel paper (Kimwipe). The phage pellet was resuspended in 300ul of buffer (50mM Tris-HCl pH 8.0, 10mM NaCl, 1mM EDTA) and transferred to a 1.5ml polypropylene tube. 300ul of buffered phenol was added and the tube vortexed, followed by a 10 min. incubation at 45°C. After vortexing again, 200ul phenol and 200ul CHCl_3 was added followed by vortexing and a

5 min. spin in a microfuge. 250ul of the aqueous phase was removed to a new tube and brought to 0.3M Na acetate. 800ul of absolute ethanol was added to precipitate the DNA at -20°C overnight. DNA was pelleted in a cold microfuge for 10 min., followed by a wash with 1ml of 80% ethanol. The tubes were dried under vacuum for 30 min., the pellet resuspended in 50-100ul of water and stored at -20°C.

DNA Manipulation.

Chromosomal DNAs were restricted for Southern blots or rescue by incubation overnight with 3 units of restriction enzyme per ug of DNA under conditions recommended by the supplier (New England Biolabs, Beverly, MA. and Boehringer Mannheim Biochemicals, Indianapolis, IN.). Partial chromosomal digestions were achieved by shortening incubation time to 1.5-5 hrs. Plasmid and M13 RF DNAs were digested for 2-4 hrs. with 10 enzyme units per ug DNA. B. subtilis chromosomal DNA adjacent to plasmid insertions was rescued after appropriate restriction digestion by incubation of dilute DNA (2ug/ml) with 0.5 Weiss units of T4 DNA ligase (Boehringer) per ml and subsequent transformation into frozen competent E. coli HB101.

Insertional plasmid pWR112 was constructed by fusing the PstI-HindIII fragment of pGR102 (Figure 1a and LaFauci

et al., 1986) containing B. subtilis 16S rDNA sequences with similarly restricted pDEB-1 (Schreier and Sonenshein, 1986), a derivative of pCED6 (Donnelly and Sonenshein, 1984) which contains a promoterless lacZ gene with its own translational signals (Figure 2). The ligation mixture was transformed into E. coli HB101 and Cmr colonies which were Aps were screened for the correct plasmid. Promoter clone pWR113 was obtained by ligating the 1.7kb PstI fragment of pGR151 (see below and Figure 3) containing the tandem ribosomal promoters of rrnJ into PstI restricted pKK232-8 (Brosius, 1984a). Transformants of E. coli HB101 able to grow in the presence of 50ug Cm/ml but not 100ug Ap/ml were screened for the proper plasmid.

Southern Analysis.

Two microgram amounts of restricted chromosomal DNA or 0.5ug of plasmid DNA were loaded per well on 0.8% (w/v) agarose (Sigma) gels (0.75% for BclI chromosomal restrictions) prepared with 1x Tris-borate EDTA buffer (TBE: 89mM Tris-HCl, 89mM Boric acid, 1mM EDTA pH 8.0). Gels were electrophoresed in 1xTBE buffer containing 0.5ug/ml ethidium bromide for 1-2 days at 30mA (5-7 days at 15mA for BclI gels). All gels had one lane of bacteriophage lambda DNA restricted with HindIII as a molecular size standard. After electrophoresis, gels were immersed in denaturing solution

(0.5M NaOH, 1.5M NaCl) for 2 hrs. with gentle shaking, followed by 2 hrs. in neutralizing buffer (1.5M NaCl, 1M Tris-HCl pH 7.5). DNA was transferred to nitrocellulose (type BA-85 ,0.45 um pore size, S+S) membranes by capillary action and then bound to the filter by baking in vacuo at 80°C for 2 hours.

Filters were prehybridized at 42°C for 2 hrs. inside heat sealable freezer bags. The prehybridization solution contained: 40% formamide (Sigma), 4xSSC (1xSSC is 0.15M NaCl, 0.015M Na₃ citrate, pH 7.0), 1% glycine, 50mM phosphate buffer pH 6.5, 250ug/ml sheared, denatured herring testes DNA (Sigma), 5x Denhardt's solution (1x Denhardt's is 0.02% ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin fraction V) (Ostapchuk et al., 1980). After removal of prehybridization fluid, filters were hybridized overnight at 42°C with between 0.5×10^6 and 4×10^6 cpm of ³²P labeled DNA probe. Hybridization solution was 40% formamide, 4xSSC, 20mM phosphate buffer pH 7.0, 100ug/ml herring testes DNA, and 1x Denhardt's solution. Filters were washed at 68°C for 2 hrs. in 4xSSC with 0.1% SDS with one change of wash solution. After drying, filters were covered with plastic wrap, placed in film cassettes with Kodak XAR-5 film and exposed at room temperature or at -70°C with Dupont Cronex Lightning Plus intensifying screens. Fragment sizes were determined from autoradiograms by measuring the migration distances and applying the relationship of Bearden, 1979.

Radioactive Probes.

Plasmid DNA, isolated fragments, or lambda DNA were labeled with ^{32}P using the random primed synthesis kit (Feinberg and Vogelstein, 1983) as directed by the supplier (Boehringer). DNA was routinely labeled to specific activities from 0.5×10^8 dpm/ug to 1×10^9 dpm/ug using ^{32}P -dCTP (3000Ci/mmol: New England Nuclear, Boston, MA.). DNA fragments for labeling were isolated by separating restriction fragments on 0.8% agarose gels using Tris-acetate buffer (TAE: 40mM Tris-acetate, 2mM EDTA pH 8.0). A slit was cut in the gel just ahead of the fragment of interest and a DEAE membrane (type NA-45, S+S) inserted. The gel was further electrophoresed for 30 min. at twice the standard running voltage and DNA bound to the DEAE membrane was purified as directed by the supplier.

DNA Sequencing.

DNA fragments from pWR113 were cloned into bacteriophage M13mp18 and mp19 (Yanisch-Perron et al., 1985) in preparation for DNA sequencing. Double stranded RF phage DNA was prepared from 50ml cultures of infected JM101 by using a scaled up version of the rapid isolation procedure described above. PstI restricted phage and plasmid DNAs were joined with T4 ligase at a 3:1 target to vector (phage)

ratio under conditions recommended by the supplier. PstI-HincII doubly restricted DNAs were also ligated at a 3:1 ratio, but 50 fold more ligase was included because of the blunt ends left by HincII. After overnight incubation at 4°C, ligation reactions were mixed with competent E. coli JM101 and allowed to incubate at 0°C for 30 minutes. After 2 min. at 42°C, 3ml of molten (45°C) top agar was added to the tube, followed by 10ul of 100mM isopropyl thio beta D-galactoside (IPTG, Sigma) and 50ul of a 2% solution in dimethylformamide of 5-bromo,4-chloro,3-indolyl, beta-D-galactopyranoside (X-gal, Boehringer). The mixture was poured onto prewarmed, thick YT plates and after solidification incubated overnight at 37°C. White plaques from recombinant phage were stabbed with a sterile toothpick which was then placed in a tube with 5ml of 2xYT and 0.2ml of log phase JM101 cells. The tubes were incubated at 37°C on a culture roller for 5-6 hrs. and were used to isolate double and single stranded phage DNA.

Sequencing of specific fragments (see Figure 4 for strategy) was done by the method of Sanger et al., 1977. Deoxy and dideoxy nucleotide triphosphates and M13 17-mer sequencing primer were from Pharmacia (Piscataway, NJ.). Solid nucleotides were dissolved to give 10mM stock solutions as described by Maniatis et al., 1972 and stored at -20°C. Mixtures of stock solutions (N^o) were made according to an Amersham protocol for sequencing using ³⁵S-

dATP (400Ci/mmol, NEN). The optimized nucleotide mixtures were: 25ul G^O+25ul 0.3mM ddGTP; 25ul A^O+20ul 0.1mM ddATP; 25ul T^O+20ul 0.5mM ddTTP; 30ul C^O+20ul 0.1mM ddCTP. Primer annealing was done in 1.5ml tubes using 5ul SS DNA template, 1ul of primer, and 1.5ul of 10x Klenow buffer (100mM Tris pH8.5, 100mM MgCl₂) in a total volume of 10ul. This mix was incubated in a boiling water bath for 10 minutes and allowed to cool slowly to room temperature (20-30 min.). 15uCi of ³⁵S-dATP and 1-2 units of Klenow enzyme (Boehringer) was added and the mix distributed into 4 aliquots of 2.5ul each in 1.5ml tubes. Reactions were initiated by placing 2ul of the appropriate N^O/ddNTP mix on the rim of the tube and centrifuging briefly in a microcentrifuge to synchronize all reactions. After 25 minutes incubation at 37°C, reactions were chased for 15 min. with 2ul of a 0.5mM mixture of all four dNTPs. Reactions were stopped by adding 4ul of formamide dye mix (0.03% xylene cyanol, 0.03% bromophenol blue, 20mM EDTA in deionized formamide) and stored at -20°C or loaded onto a sequencing gel.

Polyacrylamide gels for DNA sequencing were 6% acrylamide with 8M urea in 1xTBE buffer. Gels were prepared by mixing 33.6g urea, 12ml of a 40% acrylamide (19:1 acrylamide:bis-acrylamide) stock, 16ml of 5xTBE buffer, and water to a final volume of 80ml. Immediately prior to casting, 0.5ml of 10% ammonium persulfate (prepared fresh) and 45ul of tetramethylethylenediamine (TEMED, Kodak) was

added to begin the polymerization reaction. The gels were cast between two glass plates (33x40 cm: one plate is 1.5cm shorter) separated by 0.4mm plastic spacers (Bethesda Research Laboratories, Bethesda, MD.). Edges were sealed with plastic packing tape and plates were also held together with binder clips. Before assembly, the plates were cleaned with deionized water, 95% ethanol, and acetone, in that order. The smaller plate was siliconized by wiping 2-3ml of a 2% solution of dichlorodimethylsilane in CHCl_3 onto the plate and allowing it to evaporate in a fume hood for 10 min. After assembly of the plates and sealing, 60ml of acrylamide gel was drawn into a 60ml plastic syringe without a needle and squeezed slowly between the plates which were tilted at a 30 degree angle. When the cavity was full, the flat side of a sharkstooth comb (BRL) was inserted in the top of the gel to give an even surface upon polymerization. Gels typically were left horizontal for one hour for complete polymerization, the comb was then removed and the gel stored in an autoclave bag with moist paper towels at room temperature for up to one week.

Gels were set up by removing the bottom spacer and by insertion of the pointed side of the sharkstooth comb in the top of the gel. The comb was positioned so as not to puncture the top of the gel but to rest against it. The gel was clamped to a lucite gel stand and 1xTBE buffer added to the upper and lower buffer reservoirs. After removal of any

air bubbles at the bottom of the gel, a gel surface thermometer (International Biotechnologies Inc.) was attached and the gel was prerun for 30-60 min. at 1200 volts, 50mA (Savant high voltage power supply). When the gel reached running temperature (50-60°C) sequencing samples were heated to 95°C for 3 minutes and sample wells were rinsed out with 1xTBE from a squirt bottle to remove any urea diffusing from the gel. 2ul samples were loaded between the plastic teeth using a Hamilton microliter syringe (model 710N) equipped with a narrow needle. All samples were loaded within 10 minutes. Electrophoresis for 1.5 hrs. at 1200 volts was often followed by a second loading of the same samples to increase the resolution of large DNA fragments. All runs were stopped when the bromophenol blue dye reached the bottom of the gel. Gels were then disassembled and fixed in 10% acetic acid, 10% methanol for 20 minutes, followed by drying for 1.5-2 hrs. on a slab gel dryer (Bio-Rad) at 80°C with vacuum. The dried gel was filmed using Kodak XAR-5 33x40 cm film in a large cassette for one to two days. Films were developed manually by immersion for 5 minutes in Kodak GBX developer followed by 5 minutes in Kodak rapid fixer and hardener. The developed film was read on a light box to provide better clarity.

To sequence DNA immediately 5' to 16S rDNA sequences without subcloning, an oligonucleotide (5' GCTCGACTTGCATGTAT

3') complementary to bases 51-67 of the 16S rDNA (Green et al., 1985) was synthesized on an Applied Biosystems model 380A at the 0.2umole level. The crude product was incubated in 1ml of ammonium hydroxide at 55°C overnight in a sealed vial to deprotect the bases. 250ul aliquots were evaporated in 1.5ml polypropylene tubes in a Savant speed-vac centrifuge. Dried samples were resuspended in 100ul of 0.05M triethylammonium acetate pH 7.0 (TEA-Ac, diluted from a 1M stock consisting of 1M TEA, 1M acetic acid made to pH 7.0 with TEA) and stored at 4°C until needed. Samples were desalted using Sephadex G-50 fine (Pharmacia) gel filtration beads. Beads were swelled overnight in 0.05M TEA-Ac (20ml/g beads). A 10ml column was poured in a 10ml plastic disposable pipet plugged with glass wool. After running TEA-Ac through the column for 15 min., half of the crude oligonucleotide (500ug) was loaded on the column and allowed to enter the gel bed. 1.5 ml of buffer was collected, keeping buffer above the gel bed at all times, followed by collection of 20x300ul fractions in sterile 1.5ml tubes. The A_{260} of fractions 6 through 15 was determined by diluting 20ul of each fraction with 1ml of TEA-Ac and measuring the absorbance in a 1cm quartz 1.5ml cuvette. Fraction 12 had the peak absorbance, representing 210ug of oligonucleotide, while fractions 11 and 13 had 94ug combined. These three fractions were evaporated in a speed-vac and resuspended in 200ul of water and stored at -20°C.

lul of fraction 12 was used for annealing reactions for DNA sequencing.

Enzymatic Assays.

Beta-galactosidase

Strains with integrated pWR112 were assayed for beta-galactosidase by the method of Miller, 1972. One milliliter cell samples from cultures growing in VY, minimal, or SG medium were taken at varying Klett readings and their A_{600} determined in a Spectronic 21 spectrophotometer. After pelleting cells in a clinical centrifuge, 0.9ml of Z buffer (60mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 40mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 10mM KCl, 1mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50mM beta-mercaptoethanol, pH 7.0) was added to resuspend cells. Incubation with 250ug/ml lysozyme for 10 min. at 37°C was followed by vortexing in the presence of 0.1% Triton X-100. 0.5ml samples of this lysate was added to 0.5ml Z buffer for each assay. Reactions were initiated by the addition of 0.2ml of o-nitrophenyl beta-D-galactoside (ONPG, 4mg/ml in 100mM phosphate pH 7.0) and stopped when sufficient yellow color had developed by the addition of 0.5ml of 1M 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:

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

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

Chloramphenicol Acetyltransferase.

E. coli cells bearing derivatives of pKK232-8 were assayed for CAT activity by the spectrophotometric method of Shaw, 1975 and total protein by the method of Lowry et al., 1951. 20ml of A medium with varying supplements to affect growth rate was inoculated with 0.2ml of an overnight culture and allowed to reach a Klett of 50. The culture was pelleted and washed once with 10ml of EB buffer (50mM Tris-HCl pH 7.8, 30um dithiothreitol). Following resuspension in 3ml EB buffer, cells were lysed with 6 drops of CHCl_3 and 6 drops of 0.1% SDS with vigorous vortexing. Cell debris was pelleted and samples were assayed for CAT activity and total protein. 50 to 200ul samples were tested for CAT activity by incubation with 2ml of reaction buffer (100mM Tris-HCl pH 7.8, 0.04% dithionitrobenzoic acid, 0.1mM acetyl CoA) for 15 min. at 37°C followed by initiating the reaction with 40ul of 5mM chloramphenicol. Tubes were incubated for 15 min. at 37°C and the A_{412} read against a reagent blank including all

components except chloramphenicol. Units were calculated as per Deneer and Spiegelman, 1987 after measurement of total protein (see below). One unit produces a change of 10^{-3} A_{412} units per minute per milligram of protein.

A volume of 0.5 to 1ml of cell lysate was assayed for total protein by incubation with 6ml of alkaline copper reagent (0.01% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.02% Na tartrate, 2% Na_2CO_3 in 0.1N NaOH) in a total volume of 7.2ml. After 10 min. at room temperature, 0.3ml of Folin-Ciocalteu reagent was added and the tube mixed immediately by inversion, followed by a 30 min. incubation at room temperature. The A_{500} was determined and protein concentration determined by comparison to a standard curve constructed by using 0-300 ug of BSA under identical reaction conditions.

Alkaline Phosphatase.

Alkaline phosphatase levels of sporulating B. subtilis were measured by the method of Torriani, 1960. 0.5ml samples were pelleted and resuspended in 2ml of 1M TrisHCl pH 8.0 with 5 drops of toluene to permeablize cells. 1ml of cell suspension was mixed with 1ml of 0.1% p-nitrophenylphosphate (Sigma) and incubated until sufficient color developed, at which time the reaction was stopped with 1.5ml of 2.0N NaOH. Before reading the A_{410} , tubes were spun in a clinical centrifuge for 5 minutes after the

addition of a spatula of barium carbonate. Units are defined as the A_{410} of the sample. The alkaline phosphatase peak was at 5 hrs. after sporulation began.

Sporulation Frequencies.

Cells were also assayed for viable spores after overnight growth in SG medium. One ml aliquots of cells were heated at 80°C for 15 minutes and appropriate dilutions plated on nutrient agar.

RESULTS AND DISCUSSION.

Clustering of rRNA Operons.

It was known by the heteroduplex mapping of Chow and Davidson, 1973, that several rRNA gene sets are found in tandem on the B. subtilis chromosome. They describe a group of three gene sets separated by small spaces of 0.3 and 0.6 Kb.; the same cluster has been described by Bott et al., 1985 and Widom et al., 1988 and called the rrnI,H,G cluster. Genetic mapping of integrant strains (performed in collaboration with E. Jarvis), using the plasmid Cm^r gene shows that two insertions are found at the same location near cysA, supporting the idea of a new doublet of rRNA genes. The cotransduction frequencies of Cm^r to cysA show that insertions into the 4.9Kb. and 5.8Kb. BclI homologs (Figure 5) map to the same location in Kit1. Several other markers in the area surrounding cysA were tested, including purA, rpsE(spcF), glpT, lin-2, amyE, and aroI. The results shown in Table 3 show a "gradient" of transduction values as one moves the insertions closer to aroI and further from the origin. The data clearly establishes that rrnJ and rrnW are located at the same place, distinct from the rrnI,G,H cluster, which is farther away from the origin. As shown in Figure 6, the rrnJ,W cluster maps between purA16 and cysA14, closely linked to the latter (Jarvis et al., in press). The

rrnI,H,G cluster described above is on the opposite side of cysA14, showing linkage to aroI. As will be shown below, the clustering of rRNA genes gives rise to numerical heterogeneity.

Numerical Heterogeneity

All Bacillus subtilis strains do not contain 10 rRNA gene sets. As shown in Figure 7a, strain 168T contains 10 BclI chromosomal fragments which hybridize to rDNA probes. Since only a single BclI site is present in rDNA sequences (Green et al., 1985), each rRNA gene set, except for the 5S and 3' end of the 23S sequences, is contained on a single fragment (LaFauci et al., 1986). Strain BD170 has only 9 BclI homologs; the 4.8Kb. fragment is missing. Strain CU420 is missing the 5.8Kb. homolog.

Interestingly enough, these same BclI homologs can be deleted as a result of plasmid insertion into strain 168T (or any strain containing 10 rRNA gene sets). In a normal plasmid insertion, a single BclI homolog is displaced by a high molecular weight fragment composed of the vector plus rDNA sequences (LaFauci et al., 1986). Strains 168T-72 and 168T-77 are each missing two BclI rDNA homologs (Figure 7a) yet each shows only one high molecular weight fragment, indicating that two insertions did not occur in the same strain. Strain 168T-72 is missing the 5.5Kb. and 4.8Kb.

fragments, but only an 11.3Kb. high molecular weight fragment is found (Figure 7a). The high molecular weight fragment corresponds closely to the vector (pWR103) inserted into the 5.5Kb. fragment (vector 5.6Kb. + rrn 5.5Kb. = 11.1Kb.). If the vector had also been inserted into the 4.8Kb. homolog, a high molecular weight fragment of approximately 10.4Kb. would also be seen. In the case of 168T-77, the 5.8Kb. and 4.9Kb. homologs are replaced by a 10.6Kb. fragment, corresponding to insertion into the 4.9Kb. homolog (vector 5.6Kb. + rrn 4.9Kb. = 10.5Kb.), as opposed to the 5.8Kb. fragment (expected size = 11.4Kb.; Figure 7a). In both strains, only a single insertion took place with a concomitant deletion of rDNA sequences. Strain BD170-20 shows a similar deletion of the 5.8Kb. fragment during insertion into the 4.9Kb. homolog (Figure 7a). The 4.8Kb. fragment also absent is due to the use of BD170 as a parent strain for the insertion; the 4.8Kb. homolog is already deleted in this strain.

To confirm that rDNA had been deleted, Southern blots of EcoRI chromosomal digests were probed with 23S sequences downstream of the plasmid insertion site. Since there are two EcoRI sites 3' to the inserted plasmid (Figure 1b) and the 23S probe hybridizes downstream of these sites, no change should be seen in the fragment pattern of an integrant strain unless a deletion has occurred. Figure 7b shows 10 EcoRI fragments generated from strain 168T. The

natural deletion strains BD170 and CU420 are missing the 2.9Kb. and 3.9Kb. fragments, respectively. As with the BclI fragments, strains 168T-72 and -77 show losses of EcoRI fragments identical to BD170 and CU420. Strain 168T-72 is missing the 2.9Kb. fragment like BD170 while strain 168T-77 is missing the 3.9Kb. homolog as is CU420. The BD170 derivative (BD170-20) is missing both the 2.9Kb. and 3.9Kb. EcoRI fragments, indicating a second rDNA deletion. Since the blot was probed with vector sequences as well as 23S rDNA, integrant strains show a 5.6Kb. vector fragment.

Plasmid induced deletions of rDNA can be explained by an interaction between rrn gene clusters and multimeric integrative plasmids which exist in the cleared lysates made from E. coli. As shown in Figure 8, a double crossover event between a multimeric integrative plasmid and two closely spaced rRNA gene sets could account for all observations. In the example shown, the double crossover causes the deletion of the 2.9Kb. EcoRI fragment which spans the space between rrnH and rrnG. The BclI data is also explained by the model. The 5.5Kb. and 4.8Kb. fragments are both missing, but only a single 11.3Kb. high molecular weight fragment is seen. The rDNA which is deleted is actually the 3' end of rrnH and the 5' end of rrnG; one operon equivalent. In addition, the DNA spacer between the gene sets is deleted.

The second induced deletion involving the 4.9Kb. and

5.8Kb. BclI fragments occurs at the new cluster of rRNA genes. As described above, the rrnI,H,G cluster is the site of the deletion involving the 5.5Kb. and 4.8Kb. fragments. One could predict that a deletion might take place involving rrnI and rrnH (6.0Kb. and 5.5Kb. BclI homologs) yet this has not been seen to date. The second deletion occurs at the genetically distinct location involving rrnJ and rrnW (4.9Kb. and 5.8Kb. BclI homologs, respectively; see below). The same model can explain this deletion and therefore supports the genetic evidence that rrnJ and rrnW are in a tandem arrangement.

Spontaneous Deletions of rRNA Genes.

As shown above, plasmids only cause deletion of rDNA at two distinct locations where rRNA genes are found in tandem array. It is these exact locations where naturally occurring deletions are also found. Because of the close proximity of two rRNA gene sets, intrachromosomal recombination can take place, leading to deletion of rDNA. The model in Figure 9 shows such an event occurring involving rRNA gene sets H and G. This event probably occurred in the parent strain of BD170, where the 4.8Kb. BclI and 2.9Kb. EcoRI fragments are missing.

In order to verify this model, integrant strains SB25-40 and BD170-12 were used as sources of DNA for plasmid

rescue. Strain SB25-40 has a single insertion into rrnG and BD170-12 has a single insertion into rrnH with rrnG already missing because the parental strain was BD170. Since the enzyme EcoRI shows missing fragments in Southern blots of deletion strains, it was decided to examine the location of flanking EcoRI sites in the two insertion strains mentioned above. The integrant DNAs were partially digested with EcoRI, self-ligated at low DNA concentration, and transformed into E. coli HB101 with selection for the Tc^r of the vector. DNA from SB25-40 yielded plasmid pWR306 while BD170-12 DNA gave plasmid pWR291. Table 4 lists fragments created upon EcoRI digestion of the two plasmids.

In both plasmids, the 6.2Kb. fragment represents the vector (pGR110; Figure 1a) attached to adjacent rDNA. The 0.8Kb. piece is the small internal EcoRI fragment in the 23S gene (Figure 1b). The remaining fragments represent DNA extending from rDNA internal EcoRI sites to unique 3' and 5' EcoRI sites. Both plasmids contain a 2.3Kb. EcoRI fragment which hybridizes to 23S rDNA sequences. This proves that the EcoRI site downstream of rrnG which gives rise to the 2.3Kb. fragment of pWR306 is moved next to rrnH in strain BD170, because the rescue from BD170-12 (an rrnH insertion) gives the identical 2.3Kb. EcoRI fragment. Plasmid pWR306 shows a 2.9Kb. EcoRI fragment hybridizing to both 16S and 23S sequences. This fragment must therefore contain the 5' end of rrnG and the 3' end of rrnH. In plasmid pWR291, a

3.6Kb. EcoRI fragment is found, containing the 5' end of rrnH and the 3' end of rrnI. As shown in Figure 10a, the 2.3Kb. EcoRI fragment of both plasmids is found in all parental strains, even in BD170 which has an rrnG deletion. The 2.9Kb. fragment missing in BD170 is found at the 5' end of rrnG and the 3' end of rrnH, as demonstrated in plasmid pWR306 and Figures 10a and b. When rrnG is deleted, the 5' end of rrnH is unaltered, giving a 3.6Kb. fragment containing the rrnI 3' end and the rrnH 5' end. The model shown in Figure 9 can explain all of the observations. The EcoRI site distal to rrnG is moved distal to rrnH when rrnG is deleted. The 3.6Kb. fragment created by EcoRI containing DNA upstream of rrnH is unaltered, and the 2.9Kb. fragment normally found downstream of rrnH is deleted. An identical event can be proposed for the rrnJ-rrnW pair, which leads to the deletion of a 3.9Kb. EcoRI fragment containing the 3' end of rrnJ and 5' end of rrnW (Figure 10), as is seen in strain CU420.

The frequency of such spontaneous deletion events is unknown but one such event was observed in the course of genetic mapping of rRNA operons. Strain SB25-39 has an insertion of pGR110 into rrnD. As shown in Figure 11a, the 5.4Kb. BclI fragment is displaced, yet the 4.8Kb. fragment normally found in SB25 is also absent. Previously, the 4.8Kb. BclI homolog was only associated with the 5.5Kb. fragment when two bands were displaced. The chromosomal

location of rrnD (5.4Kb. BclI homolog) is near thiA at 70° on the genetic map (LaFauci et al., 1986), yet the 4.8Kb. rrnG fragment is found at 14° (LaFauci et al., 1986, Bott et al., 1984). It is not likely that the insertional plasmid interacted simultaneously with these two rRNA gene sets. The EcoRI blot (Figure 11b) shows that strain SB25-39 is missing the 2.9Kb. fragment associated with the spontaneous deletion that occurs between rrnH and rrnG, as in strain BD170 (Figure 7b). This spontaneous event must have occurred at some point in the culture of SB25 cells which were used during the insertion experiment that gave rise to SB25-39. It is not clear, however, whether the deletion derivative was present in the SB25 culture prior to transformation or whether the deletion event occurred during plasmid insertion into rrnD as an unrelated event. It is possible that the competent state could make such deletion events more likely, due to the induction of recombination pathways (Love et al., 1985) and the presence of recombinogenic gapped DNA in competent cells (Harris and Barr, 1971). The selection for Cm^r cells after exposure to plasmid DNA is also selecting the population of competent cells in the culture which might be more prone to create the deletion.

Heterogeneity of Regulatory Regions.

In order to examine the 5' regulatory sequences of

different rRNA operons, previously characterized insertion strains were used as a source of DNA for plasmid rescue experiments. The restriction endonuclease BclI does not have a recognition site in the insertional plasmids pGR102 or pWR103 (LaFauci et al., 1986; Figure 1a) and only a single site in rDNA sequences (Green et al., 1985; Figure 1b). Restriction of integrant DNAs was expected to release a fragment of DNA containing the inserted vector, 23S rDNA downstream of the insertion, and 16S rDNA with 5' regulatory sequences (Figure 12). The tetracycline resistance marker of the plasmid could be selected in E. coli after self-ligation of the restricted DNA and transformation. The self-ligation was carried out at low DNA concentration (2ug/ml) to avoid multi-molecular events, which might bring together DNA fragments which are not contiguous on the chromosome.

Stability of Rescued Plasmids Containing unique 5' Regions.

The presence of strong promoters on the rescue plasmid might lead to instability (Brosius, 1984b), so the Tc^r colonies were tested by colony hybridization to determine whether any sequences were rescued. The EcoRI-HindIII fragment of pYR104 (Figure 1a and Jarvis et al., in press) was used as the probe; these rDNA sequences are located downstream of the insertion site and should be

included in any successful rescue. A sample autoradiogram of a replica filter probed as above is shown in Figure 13. Colonies 1 and 2 were carrying the negative control plasmid pJH101 and colonies 3 and 4 the positive control pWR182 (a previously confirmed rescue). As can be seen for strain BD79-175, two out of eight colonies carrying Tc^r plasmids do not have expected rescue sequences, indicating that a deletion occurred in the plasmid while in E. coli. Only three out of thirteen colonies carrying rescues from BD170-143 have rescued sequences, indicating that rescues are not equally stable, possibly due to differing promoter strengths. Positive colonies from these grids were picked to grow large scale (500ml) cultures for plasmid isolation. Analysis of large scale preparations by restriction showed that confirmed rescue plasmids sometimes lost rescued sequences (Table 5). In all cases where rescued sequences were lost, analysis of the plasmid indicated that only the original insertional plasmid remained, showing that recombination was occurring between directly repeated sequences of the plasmid (Figure 14) even in recA E. coli HB101. Rescues of strains with pGR110 insertions were the most stable, with no loss of rescued sequences seen in primary rescues. This may be due to the fact that pGR110 rescues have the smallest direct repeats of the three plasmids shown. When plasmid DNA stocks were low, DNA was transformed into HB101 and resulting colonies were picked at

random to grow for large scale isolation. Table 5 shows that even stable rescues, such as those from rrnD, do not always retain rescued DNA when transformed into HB101. Finally, no transformants of rescued plasmids in recF (recA⁺) strains of E. coli retained rescued sequences (Table 5).

To confirm the rescue plasmids genetically, rescue plasmid DNA was used to transform B. subtilis to Cm^r. The first evidence that a rescue had been successful was an increase in the transformation efficiency of the rescue plasmid compared to the original integrative plasmid. As shown in Table 6, the original mapping vectors pGR102, pWR103, and pGR110, containing only operon-internal sequences, give transformation frequencies from 1.4×10^2 to 5.4×10^3 per ug DNA. The rescue derivatives of these parent plasmids generally show an increase in transformation frequency. The expected amount of homologous DNA is from 5 to 7 times greater in the rescues, which might account for the difference in frequency. An additional factor might explain the higher transformation frequency. The rescue plasmids should contain upstream non-ribosomal DNA sequences. It is possible that the pure rDNA sequences of pGR102, pWR103, and pGR110 do not afford a good pathway to integrate into the chromosome; recombination in rDNA sequences is difficult (Iglesias et al., 1983). The non-rDNA provided in the rescue plasmid might facilitate easier

synapsis during transformation. If this is true, rescued plasmids should integrate using the 5' region, thus returning to the same location as they were rescued from.

Some reintegration events were genetically mapped by PBS1 transduction. As shown in Table 7, the rescue plasmids tested did not always return to the site from where they were rescued. This indicates that the large amount of rDNA sequences in the rescues can cause integration into any rRNA gene set. The number of reintegrations into rrnD, for example, is much higher for the rescued plasmid than for the original insertional vectors. For plasmid pWR182, 14% of reinserctions mapped to rrnD, where only 6% of the original insertional vectors mapped at this locus (Table 7 and Jarvis et al., in press). The unique sequences upstream do have some influence over the site of reintegration.

Restriction Analysis of Rescued Plasmids.

To isolate unique 5' sequences from rescued plasmids, restriction analyses were carried out by using a variety of restriction endonucleases and by making use of the published nucleotide sequences of a B. subtilis rRNA operon (rrnB; Green et al., 1985) and the sequence of the vector pJH101 (Ferrari et al., 1983, K. Bott, personal communication). Since the rescues were performed using the enzyme BclI, the most straightforward approach would be to restrict with BclI

and a second enzyme which would release the 5' region, since the BclI site marks the 5' boundary of unique rescued sequences (Figure 12). A technical problem arose, since the recognition site for BclI contains the tetranucleotide sequence GATC which is methylated in all dam⁺ E. coli (Marinus and Morris, 1973), such as HB101. The adenine methylation inhibits the BclI enzyme, so the rescue plasmids had to be grown first in a dam⁻ cell in order to use BclI. As can be seen in Table 5, the instability of the rescues causes all rescued sequences to be deleted in E. coli strain GM2929, which is dam⁻ and recF13, but not recA⁻ (M. Marinus, personal communication).

Since BclI could not be used, the rescued plasmids were restricted with a series of enzymes for which the cutting sites in the vector and rDNA sequences were known. Table 8 shows the number of expected DNA fragments that would be found after restriction with various enzymes, assuming that no sites are found in the unique 5' region. If more fragments are found than expected, then additional cutting sites must be located in the 5' rescued DNA. Rescues from pWR103 integrants were expected to have one less PstI site because the PstI site found in pGR102 is absent in pWR103. A 95bp. HindIII fragment extending from 23S rDNA sequences into the vector (Figure 12) was not expected to be seen on the 0.8% agarose gels used for these analyses.

As shown in Table 8, rescue plasmid pGR151 has one more PstI site than expected, indicating a PstI-site is found in the unique rescued sequences. The single HindIII fragment appears to be two pieces of nearly identical size. Rescue pGR175 appears to have an additional HindIII site in the 5' upstream region, since the small (95bp.) HindIII fragment is not seen. Plasmid pWR179 contains extra EcoRI and PstI sites, pWR181 an extra HindIII, and pWR182 has one extra EcoRI and two additional HindIII sites.

By calculating the sizes of restriction fragments and using the published sequences of rDNA, the promoter bearing fragment could be identified and cloned using the enzymes which cut in the unique region of the rescue plasmid. Examination of the PstI-EcoRI double restrictions shown in Table 9 and comparison with Figure 12, the rDNA and vector containing fragments can be identified, leaving the fragment carrying the unique DNA. As will be shown below, the identities of several rescued fragments were confirmed by Southern blot or by DNA sequencing or by comparison to previously sequenced DNA.

Rescue pGR175 from rrnH showed evidence of a HindIII site in the 5' region. The upstream region of rrnH had been previously sequenced by Wawrousek and Hansen, 1983, and P. Zuber PhD. dissertation 1981, University of Wisconsin, and three closely spaced HindIII are found in the upstream sequences. In addition to the HindIII sites, the EcoRI

digest releases a 2.9Kb. fragment, which has been described above as containing the 5' end of rrnH and the 3' end of rrnI (Figure 10c).

A more in-depth analysis was carried out on the rrnO rescue plasmid pWR179, since the upstream region of this operon has been well characterized and completely sequenced (Moriya et al., 1985, Lampe and Bott, 1984, Lampe and Bott, 1985). The plasmid pAK1 contains an 8Kb. DNA fragment from near the replication origin of Bacillus subtilis and includes the gyrA and gyrB genes coding for the subunits of DNA gyrase (Lampe and Bott, 1985). The gyrase genes have been shown by Moriya et al., 1985, to be immediately upstream of rrnO, so the rescue plasmid pWR179 should contain the gyrA gene and 287bp. of gyrB up to the BclI restriction site (Figure 15a). The 2.2Kb. EcoRI-BamHI fragment from pAK1 containing the gyrA gene was used as a hybridization probe for Southern analysis of EcoRI-BamHI restricted pWR179. Figure 15b. shows a 2.2Kb. EcoRI-BamHI fragment common to pAK1 and pWR179, both of which hybridize to the gyrA probe. The same fragment is found in a digestion of 168T chromosomal DNA with the same enzymes. The higher molecular weight fragments in the chromosomal blot are due to incomplete restriction. Since all of the expected restriction fragments are found in pWR179 and the expected homology with gyrA found as well, it can be concluded that pWR179 is a true rescue from rrnO.

Additional genetic evidence was obtained by transforming B. subtilis strain N10 (Nal^r) with pWR179. Since N10 has a gyrA mutation conferring resistance to nalidixic acid, transformation with a normal gyrA gene will produce a Nal^s cell (Lampe and Bott, 1985). As shown in Table 10, insertional plasmid pGR102 and pWR103 containing only rDNA sequences do not give Cm^r transformants which are Nal^s. Plasmid pAK1 which carries the gyrA gene (Nal^s), transforms strain N10 simultaneously to Cm^r, Nal^s. The same is seen for rescue plasmid pWR179, confirming the presence of gyrA in this rescue. Since the rrnO rescue contains the most 5' unique DNA (3.5Kb.) of all the rescues, it was important to see whether rearrangements or deletions would occur in the DNA, but the data for pWR179 indicates that although the rescue is unstable, the upstream regions do not suffer preferential deletion or rearrangements. Plasmids rescued from integrants either contain the predicted 5' unique regions or merely the original insert.

Analysis of rrnJ Promoter Region.

The promoters of rrnJ were chosen for further study because rrnJ forms a tandem with rrnW and might have an unusual promoter which could possibly transcribe both rRNA gene sets as a single operon. The plasmid carrying the promoters (pGR151) was not stable in E. coli, so the

promoter bearing PstI fragment (1.7Kb.) was cloned into the E. coli promoter probe vector pKK232-8 (Brosius, 1984a; Figure 3). E. coli ribosomal terminators after the promoterless CAT gene protect the cell from harmful effects of high transcription levels. Transformants of E. coli able to grow in the presence of 50ug Cm/ml were picked and analyzed by restriction of mini-lysate plasmid DNA. The resultant plasmid, pWR113, was grown for large-scale plasmid isolation and restriction analysis is shown in Figure 16. The 1.7Kb. PstI fragment is inserted into pKK232-8, displacing the resident PstI fragment containing the 5' part of the Ap^r gene and most of the polylinker region. An asymmetrically located EcoRI site was used to confirm the orientation of the PstI fragment cloned in pWR113. EcoRI digestion gives a large and a small (400bp.) fragment (Figure 16), indicating the orientation shown in Figure 3. The opposite orientation would yield the same large vector fragment and an intermediate size (2Kb.) fragment.

The 1.7Kb. PstI fragment was cloned into M13mpl8 and RF DNA from white plaques analyzed for the correct fragment. A positive clone was used as a source of SS DNA for use as a sequencing template. The DNA sequence determined from the template using the M13 universal sequencing primer (17mer) is shown in Figure 17 compared to the 16S rDNA sequence near the PstI site of rrnB determined by Green et al., 1985. There are no differences in the 135 bases sequenced; this is

expected as the sequence is part of the structural gene. The last 11 bases are from the M13 polylinker and do not correspond with the 16S sequence. This analysis confirmed the identity of the PstI fragment and showed that the non-coding strand was being used as the sequencing template. Using this information and the published sequence of Green et al., 1985, a primer was synthesized which would hybridize to bases 51 to 67 of the 16S rDNA, assuming no differences in sequence between rrnB and rrnJ. This primer allowed for the sequencing of the promoter-leader region without having to sequence through 800bp. of 16S rDNA. The sequencing scheme is shown in Figure 4. The HincII site was located by sequence analysis using the synthetic primer and two PstI-HincII fragments were subsequently cloned into M13mpl8 and mpl9 and sequenced as shown.

Analysis of rrnJ Upstream Sequences.

The sequence of the rrnJ promoter region is shown in Figure 18. The 5' terminus of the 16S rDNA can be found near the end of the sequence. Two putative promoter sequences can be found, each with consensus -35 and -10 boxes separated by 17bp. as expected for ribosomal promoters of B. subtilis (Ogasawara et al., 1983). Ogasawara et al., 1983, proposed a consensus stable RNA control sequence, a seven base stretch beginning at the transcription initiation

site of stable RNA genes. This sequence begins 7 to 8 bases downstream of the -10 region. Comparison of these regions from rrnJ (both for P1 and P2) shows a good match to the consensus (G T C/T G C/T T T/Pu) with G T T G C C G found 8 bases from the -10 of P1 and G T C G C T T found 7 bases from P2. The role of this consensus sequence is not known; it has been proposed to be involved in stringent control, since it is found in front of genes coding for stable RNA, but not those coding for proteins (Ogasawara et al., 1983).

Preceding the putative P1 is the end of a possible open reading frame followed by a large stem-loop, indicative of a transcription terminator. The free energy associated with the stem-loop is -20 Kcal/mole as calculated by the method of Zuker and Stiegler, 1981. As shown in Figure 19, the sequence determined so far shows 15 amino acids followed by a UAA stop codon and then the stem-loop. This stem-loop is only 22bp. upstream of P1. A similar arrangement is found for rrn0 where 12bp. upstream of P1, the gyrA terminator is found (Moriya et al., 1985). A larger secondary structure of the DNA upstream of P1 could be formed (Figure 20), bringing sequences from -150 from the proposed P1 start site next to the -35 region. If an upstream activator is present, as in E. coli (Gourse et al., 1987), the secondary structure could bring this region close to the ribosomal promoter, perhaps allowing interaction between a bound factor and RNA polymerase at P1. Several

cases have been described of upstream activator sequences. In the tyrT promoter of E. coli, RNA polymerase can protect DNA from nuclease as far as -130 bp. from the transcription start site, if a -10 box mutant is used (Travers et al., 1983). The spoVG promoter of B. subtilis contains an AT rich sequence between -51 and -76 which, when removed, reduces the activity of the dual promoters of this sporulation gene (Banner et al., 1983). Mutations in this region can abolish spo0A dependence of spoVG transcription (Zuber and Losick, 1987), possibly by increasing the effectiveness of the activator. This AT rich region is a common feature of strong promoters in B. subtilis (Moran et al., 1982). Finally, Gourse et al., 1987, noticed that DNA from upstream of P1 in E. coli rRNA operon B runs aberrantly on agarose gels, indicating some unique structure is formed in the DNA between -50 and -80 from P1. The secondary structure predicted for the rrnJ promoter could cause analogous effects.

When one examines the P1 and P2 promoters and leader regions of B. subtilis and E. coli rRNA operons, a greater degree of heterogeneity is seen among the B. subtilis sequences. The P1 promoters of E. coli rRNA operons are all nearly identical, with variation at only three positions in three out of seven operons (Table 11). In contrast, only two out of five -35 boxes of B. subtilis P1 promoters are identical; the -10 sequences are identical for 3 out of 5.

The rrnO operon has one less nucleotide separating its -35 from -10 boxes; all E. coli operons have the same 16bp. spacing. Table 11 also shows that where E. coli has two classes of operons with respect to consensus sequences and P1 to P2 spacing, the B. subtilis operons cannot be put into categories. The P1 to P2 spacing in B. subtilis can vary from 50 to 69 bp. and the P2 to 16S spacing varies from 117 to 182 nucleotides, largely due to a 65bp. insert found in rrnO, rrnB, and rrnD (Table 11).

The sequences of six rRNA gene upstream regions from B. subtilis are compared in Figure 21. They are aligned by their 16S sequences and P2 promoters. One can see the position of the 65bp. insert occurring in rrnO, B and D immediately upstream of the 16S sequences. Upstream of this insert is a stretch of approximately 90 highly conserved nucleotides. The rest of the leader regions show varying degrees of homology; generally AT rich. The rrnH sequence does not have a P1 promoter; a cluster of 6 tRNA genes is found upstream of the single promoter (Figure 21 and Wawrousek and Hansen, 1983).

Downstream of the P2 promoters, at the beginning of the 90 bp. of high conservation mentioned above, a 16 base sequence is found which is repeated in the 16S-23S spacer region of at least rrnO and rrnB (Ogasawara et al., 1983, Green et al., 1985). This region has been proposed by Ogasawara et al., 1983, to be involved in long-range base

pairing, bringing together sequences from the 16S leader and 16S-23S spacer, as well as bringing the 16S-23S spacer sequences next to 23S downstream sequences. A complementary sequence can base pair with this conserved region, creating a cleavage site for RNA processing enzymes involved in cleaving precursor sequences to give mature rRNA (Ogasawara et al., 1983, Sogin et al., 1976). An additional role for this highly conserved sequence can be proposed based on homology with the E. coli system. Figure 22 compares the B. subtilis conserved sequence with those of E. coli rrn leaders, 16S-23S spacer, and lambda phage nut sites. In E. coli rRNA operons, this Box A-like sequence is involved in an antitermination mechanism which allows readthrough of the entire rRNA operon (Li et al., 1985, Gourse et al., 1987). Although not described in B. subtilis, it is possible that the same system exists, as many other regulatory features are conserved between the gram-negative and gram-positive bacteria.

Promoter Activity Measurements.

The expression of the dual promoters of rrnJ was examined by measuring CAT activity from plasmid pWR113 contained in E. coli HB101. As described previously, the B. subtilis promoters activate the promoterless CAT gene of pKK232-8 in pWR113, giving resistance to at least 50ug

Cm/ml. Transcriptional signals must be conserved between E. coli and B. subtilis including sequences which determine promoter strength, since high levels of CAT activity are achieved. In a previous study, the promoters of B. subtilis rRNA operon B were examined in the same way (Deneer and Spiegelman, 1987). An important feature of rRNA promoters, growth-rate regulation, was measured and found to be true of these gram-positive promoters. The sequences required for growth-rate regulation are conserved between B. subtilis and E. coli.

The dual promoters of rrnJ cloned in pKK232-8 (pWR113) were subjected to the same analysis as described in Deneer and Spiegelman, 1987, with the assistance of S. Buchholz. E. coli HB101 carrying pWR113 and several control plasmids were grown in different medium to obtain varying growth rates. Control plasmids were all derivatives of pKK232-8 containing different promoters cloned upstream of the CAT gene. Plasmid pKK351Ec. has the E. coli rrnB P1P2 promoter pair; pKK292Ec. has P1 only; pKKtet has the tetracycline resistance gene promoter from pBR322 (Deneer and Spiegelman, 1987).

The different media used were all based on A medium (Miller, 1972), with different energy sources (glucose or succinate) and different amounts of yeast extract (0.4% or 0.01%). Growth rates from 0.4 doublings/hr. (in 0.4% glucose, 0.01% yeast extract) up to 1.3 doublings/hr. (in

0.4% glucose, 0.4% yeast extract) were obtained, depending on which plasmid was being used. All cultures were grown in the presence of 50ug Cm/ml. Judging by the length of lag phase and maximal growth rates in a given medium, cells carrying pWR113 were the most affected by the presence of the plasmid. It is known that strong transcription of a replicon can cause problems for the host cell (Brosius, 1984b), so this poor growth may be indicative of the promoter strength. In fact, cells carrying pWR113 achieved the highest CAT specific activity at a lower growth rate than the E. coli ribosomal promoter controls (see below).

If one plots CAT specific activity versus doubling time, a growth-rate regulated promoter gives a line with a positive slope. As shown in Figure 23, the control plasmids pKK351Ec. and pKK292Ec. both give a positive slope with pKK292Ec. having a steeper one. Since pKK292Ec. contains only the P1 promoter, the steeper slope indicates some kind of interference of the P2 promoter, as shown by the decrease in slope of pKK351Ec. The tetracycline promoter of pBR322 is not growth-rate regulated; the slope of the plot is almost zero (Figure 23). A zero slope indicates that increases in synthesis of CAT are the same as that of general protein synthesis, since specific activity is being measured. The CAT levels for pKKtet are an order of magnitude lower than those of the ribosomal promoters, indicating that the tet promoter is much weaker. The PlP2

promoter pair of B. subtilis rrnJ gives a steep positive slope, indicating growth-rate regulation (Figure 23). The maximal specific activity is 6000, higher than the other plasmids measured. The slope (change in CAT activity vs. change in doubling time) of the curve is also the steepest (slope = 7.3), with pKK292Ec. second steepest (slope = 7), followed by pKK351Ec. (slope = 2.15). The B. subtilis promoters seem to be more efficient than the E. coli promoters, showing a higher CAT expression level and showing greater increases in expression for a given change in growth rate. If one of the promoters of rrnJ is interfering with the other, the expression of the major promoter would be even higher than indicated. In any case, the signals required for growth-rate regulation (the area surrounding the -35 region of P₁) and the characteristics which make a promoter strong in E. coli are found on the B. subtilis fragment containing the rrnJ upstream region. The sequences required for regulation are probably conserved, so B. subtilis must have control mechanisms similar to E. coli.

Expression Heterogeneity.

In order to investigate the possibility of differential usage of the ten rRNA gene sets of B. subtilis, transcriptional fusions to the E. coli lacZ gene were made by using an integrating vector. Plasmid pWR112 is based on

pDEB-1 (Schreier and Sonenshein, 1986). A PstI-HindIII rDNA fragment from pGR102 (Figure 1a) was cloned into the same sites on pDEB-1 (Figure 2). Just downstream of the HindIII site are E. coli trp operon sequences which provide translational start signals. The lacZ gene is fused to the trp sequences and a CAT gene is present to provide drug selection in B. subtilis. Since pWR112 cannot replicate in B. subtilis, it must integrate into rRNA genes by a Campbell-like mechanism in order to give Cm^r transformants. This places the lacZ gene in the chromosome downstream of resident rRNA promoters (Figure 24). Transcription from the ribosomal promoters should give rise to a mRNA for lacZ which in turn can be translated to make beta-galactosidase, which can be easily assayed. There are no promoter sequences between the ribosomal promoters and the lacZ sequences, so beta-galactosidase levels should be directly related to the transcriptional activity of the rRNA operon.

Integration of pWR112.

To ascertain the sites of integration of pWR112, Southern and transduction analysis were performed as was previously done for integrations of pGR102 and pWR103 (LaFauci et al., 1986). PBS1 transducing lysates were made using the integrant strains as donors and lysates were crossed with the kit of mapping strains of Dedonder et al.,

1977. The transduction data is shown in Table 12. The cotransduction frequencies indicate that the genetic locations correspond to those previously reported for rRNA gene sets (LaFauci et al., 1986, Jarvis et al., in press). For strain SB25-96, genetic data is not sufficient to distinguish which rRNA gene set is involved, due to the close proximity of rrnJ and rrnW. Physical data from Southern blots identifies this insertion unambiguously as being in rrnW (see below). To date, out of 110 insertions genetically mapped, no insertions of pWR112 were obtained into rrnJ, rrnH, or rrnG, suggesting that these locations cannot be inserted into and give a viable cell. This could be due to an excess of beta-galactosidase production, since regular mapping plasmids (Figure 1a) have inserted into these locations many times (LaFauci et al., 1986, Jarvis et al., in press).

Chromosomal DNA isolated from integrant strains was restricted with BclI, electrophoresed in 0.75% agarose, blotted and probed with ³²P- labeled 23S rDNA. As seen before, in each case a parental rDNA homolog is missing, replaced by a higher molecular weight species consisting of vector plus rDNA sequences (Figure 25). In the case of SB25-96, the 5.8Kb. homolog is missing, identifying the site of integration as rrnW, to confirm the genetic data. Separate insertions can also be identified in rrnO (SB25-42, SB25-191), rrnA (SB25-41), rrnE (SB25-77), rrnD (SB25-43),

and rrnB (SB25-100), based on missing BclI homologs (Figure 25). In contrast to insertions of other integrable plasmids (pGR102, pWR103, pGR110; see above), the high molecular weight fragments seen do not correspond to the vector (13.5Kb.) plus ribosomal homolog. For example, strain SB25-42 would be expected to show a 21.8Kb. BclI fragment (13.5Kb. + 8.3Kb. rrnO); a 14.3Kb. fragment is found instead. The same 14.3Kb. fragment is found for SB25-41, 77, and 100, strains with insertions into three different rRNA gene sets (rrnA, E, and B respectively). These would normally give rise to three different size fragments. It is apparent that a BclI site is present in the vector, so the same size fragment is produced regardless of the operon where the insertion occurred. Figure 24 shows that a 23S probe would hybridize to fragments of a similar size in any insertion. The variable BclI site 5' to each gene set gives rise to a fragment with only 16S and vector homology, not seen by the 23S probe. When a 16S probe is used, the BclI homolog of the parental strain is displaced by a hybrid fragment of rDNA and vector sequences which is nearly the same size as the parental fragment. A second, high molecular weight fragment is also seen, identical in size to the fragment with 23S homology. Figure 26 shows strain SB25-42 having a BclI fragment with 16S homology which is 8.2Kb., only slightly smaller than the parental 8.3Kb. homolog. A second high molecular weight fragment (14.3Kb.)

is the same fragment to which the 23S probe hybridizes.

The model of integration in Figure 24 can explain the BclI patterns seen for strains SB25-41,42,77, and 100, but does not account for abnormalities seen for SB25-43,96, and 191. The 23S probe hybridizes to BclI homologs which are larger than expected. Strains SB25-96 and 191 show fragments of 18.6Kb. and 18.1Kb. respectively, while SB25-43 has a 21Kb. homolog (Figure 25). This indicates that some DNA has been duplicated and thus a larger fragment is seen. The amount of extra DNA is approximately 4Kb. for SB25-96 and 191, and 7Kb. for SB25-43.

In order to further determine where duplications might be occurring, integrant DNAs were digested with EcoRI, electrophoresed through 0.8% agarose, blotted and probed with ³²P labeled pDEB-1, containing no rDNA sequences. For a Campbell-like insertion, two fragments would be expected to hybridize to the probe, one of 5.5Kb. (constant) and a second of approximately 10.3Kb. (Figure 24). As shown in Figure 27, all integrant DNAs have a 5.5Kb. EcoRI fragment with vector homology, indicating that the operon-vector fusion at the 5' end is not altered from what is expected from a Campbell-like insertion. The vector EcoRI site is near the end of the lacZ gene, so the rrn-lacZ fusion is intact in all integrant strains. A second, higher molecular weight fragment can be found in the strains; this is the 3' side of the insertion. In agreement with the BclI data,

strains SB25-41,42,77, and 100 all contain the expected 10.3Kb. fragment, indicating no duplicated DNA. The strains showing extra sequences also have larger EcoRI fragments, SB25-96 and 191 showing 13.7Kb. and 14.5kb. EcoRI fragments, an increase over the expected 10.3Kb. corresponding to the amount that the BclI fragments exceed their expected size (see above). Strain SB25-43 shows a 17.2Kb. EcoRI fragment, which is 7Kb. larger than expected, the same amount that the BclI fragment is oversized. Whatever DNA is duplicated, it must have occurred in the vector's 3' end, after the EcoRI site. It is possible that the CAT gene has amplified to provide enough resistance to Cm or some plasmids have had duplications introduced during growth in E. coli. The plasmid does not grow well in E. coli, and transforms B. subtilis very poorly, so it is possible that some selection for duplications is being applied by trying to transform B. subtilis. The lacZ fusions seem to be unaffected by the extra DNA; evidence will be given below that fusions in the same operon give the same levels of beta-galactosidase regardless of the Southern blot pattern. As long as the 5' side of the insertion is unaltered, expression of lacZ should not be affected by distal duplications.

Expression of Individual rRNA Gene Sets During Vegetative Growth.

By using the rrn-lacZ fusions, activity levels of different rRNA gene sets was measured by determining beta-galactosidase levels under different growth conditions. Cells growing in Spizizen minimal medium (Anagnostopoulos and Spizizen, 1961) were assayed for enzyme activity (Miller, 1972) and the remainder of the culture shifted to rich medium (VY), where a second measurement was made. The shift-up was done to avoid starting the VY experiment with cells carrying multiple chromosomes; the cells growing in minimal medium should not contain multiple replication forks. This might be important because operons closest to the origin of replication (rrnO and rrnA) might be present in multiple copies compared to more origin-distal operons (rrnE and rrnD; Figure 6).

As shown in Table 13, the background level of beta-galactosidase in integrant strain 168T-87 is approximately 3 Miller units in VY or minimal medium; this strain has no lacZ gene inserted. Strain SB25 carrying pFW2, a plasmid similar to pDEB-1 with the promoterless lacZ construct, gives slightly higher beta-galactosidase activity than background. This indicates that the lacZ gene is not active unless a promoter is placed in front of it. Strain SB25-92 was created by transformation of SB25 with a ligation mixture containing pDEB-1 and B. subtilis leucine operon sequences from plasmid RSP2124 B. leu (Nagahari and Sakaguchi, 1978). The higher levels of beta-galactosidase

activity indicate that a promoter is now in front of the lacZ gene. This strain shows beta-galactosidase levels from a non-ribosomal promoter.

When one examines the beta-galactosidase levels produced in rRNA insertion strains, most measurements are higher than the control strains in a given medium. The maximal beta-galactosidase levels achieved by ribosomal insertions are 3 fold higher than the leucine insertion. The operons can be grouped according to their expression levels in VY; high (rrnO, rrnW), medium (rrnA, rrnE), and low (rrnD, rrnB). The groupings change slightly in minimal medium, with rrnE now higher than rrnW. The data cannot be explained totally based on the chromosomal position of the operons. Since rrnO is closest to the chromosomal replication origin, it might be present in more than one copy relative to the other gene sets, therefore a higher level of beta-galactosidase would be expected. However, rrnW shows a higher expression level than rrnA and is further from the origin. In addition to the case of rrnW, the minimal medium results are mostly consistent with the VY; few multiple replication forks are expected in these cells. The results in Table 13 also indicate that independently isolated insertions into the same rRNA operon give similar beta-galactosidase levels, indicating that different insertion events do not give different expression patterns.

The ribosomal-lacZ insertions respond to growth-rate regulation, giving higher levels of beta-galactosidase after shift-up from minimal medium to VY. The increase in beta-galactosidase levels are from 2 to 4 fold, as compared to only 1.3 fold for the leucine control. The elevation seen for the leucine control is approximately the same as the other control strains (Table 13).

Expression of Individual rRNA Gene Sets During Sporulation.

In order to assess the roles of individual rRNA gene sets after exponential growth, the lacZ fusion strains were grown in sporulation medium and beta-galactosidase measurements made each hour after exponential growth ceased. To insure that the insertions were not interfering with sporulation processes, the production of alkaline phosphatase was assayed as well as beta-galactosidase, in collaboration with A. White. In addition to the enzymatic assays, the number of viable spores was measured for each culture after overnight incubation in SG medium and heating at 80°C for 15 minutes, which should kill all but spores. As shown in Table 14, the number of viable cells/ml after heating varies between 2.2×10^8 and 6.6×10^8 per ml. None of the strains showed any sporulation defects where the viable counts would be reduced by at least an order of magnitude. The peak alkaline phosphatase levels, measured

as absorbance of paranitrophenol at 410nm, occur at five hours into the sporulation process. These values vary between 0.4 and 1.1, but show a consistent strain variability.

The levels of beta-galactosidase measured at different times from different rRNA gene sets are shown in Figure 28. In general, all rRNA gene sets show a decrease in activity following the end of exponential growth. The expression levels off after T_3 , with rrnO,A,W, and E giving close to 30 Miller units (Figure 28a), and the others (rrnD,B, leucine) approximately 15 units (Figure 28b). The gene sets which give higher expression levels during vegetative growth (see above) continue to express at higher levels during sporulation and plateau at similar levels after T_3 .

No dramatic changes in expression of rRNA genes seems to occur when the cell ceases exponential growth and enters sporulation, but some operons seem to be more important than others. Presumably, ribosomes which exist at the end of exponential growth are replaced as they degrade; protein synthesis is still needed to make new sporulation proteins. Testa and Rudner, 1975, demonstrated that ribosomal RNA synthesis continues after growth stops and can constitute a large part of total RNA. The beta-galactosidase levels produced by rrn-lacZ fusions indicate that this rRNA synthesis is not identical from all operons; certain gene

sets are responsible for larger fractions of the synthesized RNA. It is possible that, since the same gene sets are more highly expressed during vegetative growth as well, the levels seen are due to the basic promoter strength of the operon. It is not clear why one operon may have a stronger promoter than another. Promoter strength might be related to the transcriptional units, with some rRNA operons containing tRNA genes in the 16S-23S spacer or at the 3' end. A larger transcriptional unit may require a stronger promoter, such as the rrnJ, rrnW or rrnI, H, G clusters.

CONCLUDING REMARKS

Although the beta-galactosidase measurements suggest that rrn operons of B. subtilis are not transcribed equally, alternate possibilities can still be suggested. Some evidence is available to dispute the theory that chromosomal position of the operons can explain expression levels based on copy number, but this has not been totally ruled out. By making use of temperature sensitive mutants in DNA replication (dna strains; Winston and Sueoka, 1980), differences in rrn copy number can be minimized. Beta-galactosidase levels at the permissive temperature can be compared to levels at the restrictive temperature to see if copy number plays an important role. If copy number does play a role, this would mean that B. subtilis could adjust the relative rRNA contributions of individual operons by controlling chromosomal replication. Since 7 out of 10 operons are located within the first 14° from the replication origin (LaFauci et al., 1986, Jarvis et al., in press), initiation of a new round of DNA replication prior to completion of the first could rapidly increase the number of rRNA gene sets in a cell. Multiple replication forks, which do occur in cells growing in rich medium (Oishi et al., 1964), would increase the number of rRNA genes in an average cell from 10 to 17 (when the first 14° of the chromosome has been replicated). This would increase the

number of primary transcripts available to fast growing cells.

The question of message stability could be addressed as well. Differences in expression levels might be due to the half-life of lacZ mRNA from different operons. Pulse-labeling of RNA, followed by rifampicin treatment, extraction, and hybridization to lacZ specific probes would determine the message stabilities of rrn-lacZ fusion transcripts.

The sequence heterogeneities seen among B. subtilis rRNA gene sets could lead to differential rates of rRNA processing, thus modulating the contribution of individual gene sets post-transcriptionally. The three operons which have a 65bp. insert in the leader region (rrnO,B,D), might be processed at a different rate than other operons. Heterogeneities in the 23S-5S spacer region could also influence processing rates.

If true differences exist in promoter strength, further studies would be needed to determine why one operon should be transcribed more than another. Besides the obvious sequence differences of promoter regions, the makeup of the transcriptional units might provide a reason why a particular operon may need greater levels of expression. As described by Vold, 1985, many tRNA determinants are clustered near rRNA genes; possibly belonging to large transcriptional units. The cluster of tRNA genes downstream

of rrnB seems to have an internal promoter (Vold and Green, 1986), but read-through transcripts from rrnB is necessary to express the tRNA^{Val} and tRNA^{Thr} which are 5' to the putative tRNA promoter. Also, read-through transcription from rrnB may be necessary to maintain basal levels of these tRNA species. The single rRNA operon which contains the minor 5S rRNA species might require greater transcriptional levels if the minor 5S molecule plays an important role in regulation. This operon also contains a cluster of 16 tRNA genes which may have a promoter in the 23S-5S spacer region (Vold, 1985). The triplet of rRNA gene sets (rrnI, H, G) may have only one major promoter and only minor promoters in the intergenic spaces; the same might be true of the rrnJ, W doublet. The single promoters which lie between tandem repeats could be tested to see if they have all of the properties of a P₁ promoter or whether regulation is achieved through the action of a single promoter in front of the rrn cluster. These P₁ promoters may have a need to be stronger in order to transcribe a large operon.

Perhaps the best way to study rRNA regulation would be to make rrn-lacZ or rrn-CAT fusions and insert them into the B. subtilis chromosome or put them on a plasmid replicon, such as pCED6 (Donnelly and Sonenshein, 1984) and measure expression levels. Variables such as chromosomal location and insertional rearrangements could be eliminated. Mutational studies of promoter regions are feasible since

the sequences of at least 7 rrn upstream regions are known. Studies which have proven successful in E. coli (for example Gourse et al., 1987, Li et al., 1984) could be done in B. subtilis to answer similar questions, as well as the role of rRNA operons during sporulation and germination.

SUMMARY

1. A new doublet of rRNA gene sets, called rrnJ-rrnW, has been located between purA and cysA, closely linked to the latter.
2. Examination of strains by Southern analysis has shown that deletion of rDNA during plasmid insertion into the chromosome occurs only at the sites of tandemly arranged gene sets, believed to be due to a double crossover between two gene sets and a multimeric integrative plasmid.
3. Two spontaneous deletion events occur at the sites of tandemly arranged rRNA gene sets, leaving only 9 rrn operons. Only rrnW or rrnG are deleted this way, the same two which can be deleted during plasmid insertion.
4. Rescue of chromosomal DNA from the sites of spontaneous deletions indicates that intrachromosomal recombination between closely spaced rRNA gene sets is the most likely mechanism for the deletions.
5. The upstream regulatory sequences from 6 rRNA gene sets have been cloned by plasmid rescue from the DNA of strains with inserted mapping plasmids. The instability of these plasmids indicates strong promoter activity.

6. Sequence analysis of the upstream region of rrnJ show a typical rRNA promoter arrangement, with dual promoters and a leader preceeding the 16S gene.
7. The end of an open reading frame followed by a possible transcription terminator is found only 22bp. upstream of the rrnJ P1 promoter. A larger secondary structure can be formed, bringing sequences from -150 close to -50.
8. The rrnJ P1P2 promoter pair is growth-rate regulated in E. coli, as demonstrated by measurements of CAT activity under different growth conditions of cells carrying promoter-CAT fusion plasmid pWR113.
9. Insertional plasmid pWR112 was used to create rrn-lacZ fusions in the chromosome of B. subtilis.
10. Measurements of beta-galactosidase activity from integrant strains grown in VY or MM show three levels of activity; strong (insertions into rrnO or rrnW), intermediate (rrnA, rrnE), and weak (rrnD, rrnB).
11. The same strains measured during sporulation show decreasing activity, reaching a plateau at T₃, with insertions into rrnO, rrnA, rrnW, and rrnE leveling off at similar beta-galactosidase activities, and rrnB and rrnD reaching lower levels.

12. The most origin-proximal operon (rrn0) is the most active at all times during vegetative growth and sporulation, possibly due to chromosomal branching at the replication origin.

Table 1. Strains Used.

<u>B. subtilis</u>		
<u>Parental Strains</u>	<u>Genotype</u>	<u>Source</u>
168T	trpC2	K. Bott
BD170	trpC2, thr-5	D. Dubnau
BD79	leuB1, pheA1	D. Dubnau
BD29	leuB1, argA2	D. Dubnau
E88	trpE46, hisH2	J. Kane
SB25	trpC2, hisH2	D. Dubnau
CU420	trpC2, leuB6, ilvC4	S. Zahler
<u>Mapping Strains</u>		
Kit1 to Kit9	mapping strains ^a	D. Dubnau
1A221	lin-2	BGSC ^b
1A241	cysA14, rpsE2	BGSC
1A429	trpC2, aroI906, glpT6	BGSC
1A474	trpC2, amyE, aroI906	BGSC
RJ4	trpC2, amyE, lin-2, aroI906	R. Rudner
RJ13	trpC2, lin-2, glpT6, aroI906	R. Rudner
RJ25	trpC2, rpsE2, glpT6, aroI906	R. Rudner
<u>Integrants</u>		
168T-37	trpC2, Cm ^r :rrnG	This study
168T-72	trpC2, Cm ^r :rrnH	This study
168T-77	trpC2, Cm ^r :rrnJ	This study
168T-79	trpC2, Cm ^r :rrnH	This study
168T-80	trpC2, Cm ^r :rrnW	This study
168T-87	trpC2, Cm ^r :rrnA	This study
168T-88	trpC2, Cm ^r :rrnJ	This study
SB25-35	trpC2, hisH2, Cm ^r :rrnO	This study
SB25-39	trpC2, hisH2, Cm ^r :rrnD	This study
SB25-40	trpC2, hisH2, Cm ^r :rrnG	This study
SB25-41	trpC2, hisH2, Cm ^r :rrnA, LacZ ⁺	This study
SB25-42	trpC2, hisH2, Cm ^r :rrnO, LacZ ⁺	This study
SB25-43	trpC2, hisH2, Cm ^r :rrnD, LacZ ⁺	This study
SB25-74	trpC2, hisH2, Cm ^r :rrnO, LacZ ⁺	This study
SB25-77	trpC2, hisH2, Cm ^r :rrnE, LacZ ⁺	This study
SB25-80	trpC2, hisH2, Cm ^r :rrnD, LacZ ⁺	This study
SB25-92	trpC2, hisH2, Cm ^r :leu, LacZ ⁺	This study
SB25-96	trpC2, hisH2, Cm ^r :rrnW, LacZ ⁺	This study
SB25-100	trpC2, hisH2, Cm ^r :rrnB, LacZ ⁺	This study
SB25-191	trpC2, hisH2, Cm ^r :rrnO, LacZ ⁺	This study

Table 1 continued

BD170-12	trpC2, thr-5, Cm ^r :rrnH	This study
BD170-20	trpC2, thr-5, Cm ^r :rrnH	G. LaFauci
BD170-143	trpC2, thr-5, Cm ^r :rrnA	G. LaFauci
BD170-144	trpC2, thr-5, Cm ^r :rrnW	G. LaFauci
BD170-151	trpC2, thr-5, Cm ^r :rrnJ	G. LaFauci
BD170-179	trpC2, thr-5, Cm ^r :rrnO	G. LaFauci
BD170-181	trpC2, thr-5, Cm ^r :rrnA	G. LaFauci
BD79-175	leuB1, pheA1, Cm ^r :rrnH	G. LaFauci
BD79-182	leuB1, pheA1, Cm ^r :rrnD	G. LaFauci

E. coli Strains

HB101	hsdS20, recA13, aro-14, proA2, lacY1, galK2, rpsL20, xyl-5, mtl-1, supE44	K. Bott
JM101	lac, pro, thi, supE, F' traD36 proAB, lacI ^q , Z M15	E. Dubnau
GM2929	dam-13::Tn9, dcm-6, hsdR2, recF143, galK2, galT22, ara-14, lacY1, xyl-5, leuB6, thi-1, tonA31, rpsL136, hisG4, tsx-78, mtl-1, glnV44	M. Marinus

a) Mapping strains are described by Dedonder et al., 1977.

b) Bacillus Genetic Stock Center

Table 2. Plasmids Used

<u>Plasmids</u>	<u>Relevant Genes</u>	<u>Source</u>
pJH101a	Ap ^r , Tc ^r , Cm ^r	K. Bott
pKK232-8	Ap ^r , Cm ^s	D. Morrison
pDEB-1 ^b	Ap ^r , Cm ^r , lacZ	A. Sonenshein
pFW2	Ap ^r , Km ^r , T7 term. lacZ	A. Sonenshein
pGS227	Ap ^r , galK, Plp2 rrnB <u>B. subtilis</u>	K. Bott
pAK1	Ap ^r , gyrA	K. Bott
pKK292Ec.	Ap ^r , Cm ^r , Pl rrnB <u>E. coli</u>	G. Spiegelman
pKK351Ec.	Ap ^r , Cm ^r , Plp2 rrnB <u>E. coli</u>	G. Spiegelman
pKKtet	Ap ^r , Cm ^r , P tet	G. Spiegelman
pGR102a	Tc ^r , Cm ^r , 16S-tRNA-23S	G. LaFauci
pGR110a	Tc ^r , Cm ^r , 16S	G. LaFauci
pWR103a	Tc ^r , Cm ^r , 16S-23S	R. Widom

Constructed Plasmids

pWR112 ^b	Cm ^r , lacZ, 16S-tRNA-23S	This study
pWR113 ^c	Cm ^r , Plp2 rrnJ <u>B. subtilis</u>	This study

Rescued Plasmids

pWR179	Tc ^r , Cm ^r , rrnO, gyrA	This study
pWR181	Tc ^r , Cm ^r , rrnA	This study
pGR151	Tc ^r , Cm ^r , rrnJ	This study
pGR175	Tc ^r , Cm ^r , rrnH	This study
pWR182	Tc ^r , Cm ^r , rrnD	This study
pWR291	Tc ^r , Cm ^r , rrnH	This study
pWR306	Tc ^r , Cm ^r , rrnG	This study

a) See Figure 1a.

b) See Figure 2.

c) See Figure 3.

Table 3. Cotransduction Frequencies of Cm^r Plasmids in rrn Operons Near the Replication Origin.^a

<u>Operon</u>	<u>BclI</u> <u>frag. (Kb)</u>	<u>Donor</u>	<u>Cm</u> <u>Pur</u>	<u>Cm</u> <u>Cys</u>	<u>Cm</u> <u>Spc</u>	<u>Cm</u> <u>Glp</u>	<u>Cm</u> <u>Lin</u>	<u>Cm</u> <u>Amy</u>	<u>Cm</u> <u>Aro</u>
rrnO	8.3	SB25-35	61	41	33	6	0	0	0
rrnA	5.7	168T-87	46	51	46	10	0	0	0
rrnJ	4.9	168T-88	39	80	58	31	9	3	0
rrnW	5.8	168T-80	41	75	60	27	10	3	0
rrnH	5.5	168T-79	5	67	73	48	32	28	12
rrnG	4.8	168T-37	7	64	76	42	28	35	12

a) Values are cotransduction percent with PBS-1. The recipient strains were: Kit 1 (trpC2, purA16, cysA14), 1A241 (cysA14, rpsE2), RJ4 (trpC2, amyE, lin-2, aroI906), RJ13 (trpC2, lin-2, glpT6, aroI906), and RJ25 (trpC2, rpsE2, glpT6, aroI906).

Table 4. EcoRI Rescues of rrn Operon Flanking Regions.

Plasmid and <u>rrn</u> <u>operon</u> ^a	EcoRI fragments (Kbp.)	Hybridization ^b	
		<u>5'</u> <u>16S</u>	<u>3'</u> <u>23S</u>
pWR306, <u>rrnG</u>	6.2	-	-
	2.9	+	+
	2.3	-	+
	0.8	-	-
pWR291, <u>rrnHC</u>	6.2	-	-
	3.6	+	+
	2.3	-	+
	0.8	-	-

a) The inserted plasmid was pGR110 (see Figure 1a)

b) Probes used were a 1.9Kb. EcoRI fragment from pGS227 (Stewart and Bott, 1983) containing the 5' end of the 16S gene and a 1.2Kb. EcoRI-PstI fragment from pYR104 containing the 3' end of the 23S gene.

c) The integrant strain is missing rrnG by spontaneous deletion.

Table 5. Stability of Rescued Plasmids.

<u>Operon</u>	<u>Sucess/Fail^a</u>		<u>Sucess/Fail</u>	
	<u>Primary Transformants^b</u>		<u>Secondary Transformants^c</u>	
	pGR102 pWR103	pGR110	HB101	GM2929
rrnD	2/2	1/1	5/8	0/12
rrnJ	1/2	- ^d	1/2	0/3
rrnO	1/2	-	-	-
rrnA	1/4	-	0/1	-
rrnW	0/3	-	-	-
rrnH	1/1	1/1	-	-
rrnG	-	3/3	-	-

a) Number of large scale plasmid preparations retaining rescued sequences/ number of preparations deleting all rescued DNA.

b) Primary transformants shows plasmid preparations made from individual colonies which were positive for colony hybridization. Plasmids were extracted as described in Materials and Methods.

c) Secondary transformants shows plasmid preparations made from the indicated E. coli strains transformed by confirmed rescue plasmid DNA.

d) - = not done.

Table 6. Transforming Efficiencies in Bacillus subtilis of Integrable and Rescue Plasmids.

<u>Parental Plasmids</u>	<u>B. subtilis DNA</u> <u>Kb./ (rrn operon)</u>	<u>Cm^r Transformants</u> <u>per ug DNA</u>
pGR102	1.2	5.4×10^3
pWR103	1.0	2.8×10^3
pGR110	0.8	1.4×10^2
<u>Rescued Plasmids</u>		
pGR151	6.1/rrnJ	6.0×10^3
pGR175	6.7/rrnH	7.4×10^3
pWR179	9.3/rrnO	3.9×10^3
pWR181	6.7/rrnA	5.8×10^3
pWR182	6.4/rrnD	2.0×10^3
pWR306	5.6/rrnG	7.6×10^3

a) Several recipient strains were used including BD170 (trpC2, thr-5), BD79 (leuB1, pheA1), BD29 (leuB1, argA2), and E88 (trpE46, hisH2)

Table 7. Chromosomal Location of Integrated Plasmids.

Primary Insertions.^a

<u>rrn Operon</u>	<u>% of Total Mapping to this Operon^b</u>
rrnO or A	39
rrnJ or W	30
rrnD	6

Rescue Insertions.

<u>Plasmid</u>	<u>rrn operon</u>	<u># examined^c</u>	<u>% returning to original location^d</u>
pWR179	rrnO	28	37 ^e
pGR151	rrnJ	14	46 ^f
pWR182	rrnD	77	14

a) Insertions of integrable mapping vectors (Figure 1a) into specific locations.

b) Percent out of 190 insertions mapped genetically by PBS1 transduction which are found at the indicated location.

c) Number of Cm^r transformants from rescue plasmid DNA that were picked and used as donors for PBS1 transduction.

d) Percent of phage lysates which showed linkages of Cm^r to chromosomal markers similar to the original insertion strains.

e) The proximity of rrnA to rrnO does not allow the distinction between these locations to be made without very careful measurements. Some of these reinsertions could be into rrnA; the minimum percentage reinserting into rrnO is 18.5%.

f) Since rrnJ is a member of a tandem with rrnW, it cannot be determined by transduction which of the two gene sets has been reinserted into. The minimum percentage of rrnJ reinsertion would be 23%, if half of the observed cases inserted in rrnW.

Table 8. Predicted and Actual Number of Restriction Fragments for Rescue Plasmids.

Predicted^a

<u>Enzyme</u>	<u>pGR102</u>	<u>pWR103</u>
EcoRI	4	4
PstI	3	2
SmaI	4	4
HindIII ^b	2	2

Actual^c

<u>Enzyme</u>	<u>pGR151</u>	<u>pGR175</u>	<u>pWR179</u>	<u>pWR181</u>	<u>pWR182</u>
EcoRI	4	4	5	4	5
PstI	4	3	3	2	2
SmaI	4	4	3*	4	4
HindIII	1*	3	2	3	4

a) Number of expected restriction fragments from rescues of pGR102 and pWR103 inserts based on the known sequences of rDNA and vector assuming no sites are present in the unique rescued DNA.

b) One HindIII fragment (95bp.) would not be seen in restriction analysis and is not counted here.

c) pGR rescues were from pGR102 insertions and pWR rescues from pWR103 insertions. The number of fragments were determined by ethidium bromide fluorescence of restricted DNA separated on 0.8% agarose gels. An asterisk indicates one of the fragments found with the indicated enzyme appears to represent two fragments of identical size.

Table 9. Fragments Produced by Restriction Endonuclease Digestion of Rescued Plasmids.^a

<u>Enzyme</u>	<u>A</u>	<u>B</u>	<u>C</u>	<u>D</u>	<u>E</u>	<u>F</u>	<u>Sum of fragments</u>
pWR179 (rrnO)							
EcoRI	5.6	4.4	1.7	1.1	0.8		13.6
PstI	8.0	4.0	1.1				13.1
EcoRI-PstI	5.6	2.2	1.7 ^c	1.1 ^b	1.0	0.8	13.5
SmaI	5.3 ^b	2.3	0.7				13.6
HindIII	8.0	5.6					13.6
pWR181 (rrnA)							
EcoRI	5.6	2.8	1.2	0.8			10.4
PstI	9.0	2.3					11.3
EcoRI-PstI	5.6	2.1 ^c	1.2	1.1	0.8		10.8
SmaI	5.6	2.4	2.3	0.7			11.0
HindIII	5.6	2.5	2.1				10.2
pGR151 (rrnJ)							
EcoRI	5.6	3.4	1.2	0.8			11.0
PstI	5.6	2.9	1.7 ^c	0.85			11.1
EcoRI-PstI	4.6	1.25 ^c	1.1	1.0 ^b	0.85	0.8	10.6
SmaI	5.8	2.5	2.3	0.5			11.1
HindIII	5.6 ^b						11.2
pGR175 (rrnH)							
EcoRI	5.6	2.9 ^c	1.2	0.8			10.5
PstI	5.6	2.5	2.4				10.4
EcoRI-PstI	4.6	2.3 ^c	1.2	1.1 ^b	0.8		11.1
SmaI	5.6	2.4	2.3	0.5			10.8
HindIII	5.6	2.5	2.1				10.2

Table 9. continued.

pWR182 (rrnD)							
EcoRI	5.2	3.4	1.2	1.1	0.8	11.7	
PstI	9.2	2.5				11.7	
EcoRI-PstI	5.2	2.3 ^C	1.2	1.1	1.0	0.8	11.6
SmaI	6.4	2.6	2.2	0.5			11.7
HindIII	5.2	2.7	2.4	1.2			11.5

a) All values in kilobase pairs, determined by measuring migration in agarose and applying the relationship of Bearden, 1979.

b) Bands appear to be doublets and are counted twice in the total size.

c) Fragments containing the promoter sequences.

Table 10. Transformation of rrnO Rescue Plasmid
into Strain N10 (Nal^r)

<u>Plasmid</u>	<u># of Cm^r Transformants</u>	<u>% Nal^r or Nal^sa</u>
pGR102	2.19 x 10 ³	100 Nal ^r
pWR103	1.14 x 10 ³	100 Nal ^r
pAK1	5.0 x 10 ⁴	100 Nal ^s
pWR179 Ib	2.1 x 10 ⁴	100 Nal ^s
pWR179 II	3.6 x 10 ³	88 Nal ^s

a) Percentage of Cm^r colonies which grew (Nal^r) or did not grow (Nal^s) after replica plating to plate containing 20ug/ml naladixic acid.

b) Plasmid pWR179 was used for two separate experiments.

Table 11. Promoter Regions of B. subtilis and E. coli rRNA Operons.

<u>Operon</u>	<u>P1</u>				<u>P2</u>				<u>m16S</u>
	-35	bp. ^a	-10	bp.	-35	bp.	-10	bp.	
<u>B. subtilis</u> ^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 ^c	--	-	--	-	TTGACT	17	TATACT	122	TTTAT
rrnD	TTGACT	17	TATATT	68	TTGACA	17	TATATT	182	TTTAT
rrnB	TTGCAA	17	TATATT	63	TTGACA	17	TATATT	178	TTTAT
<u>E. coli</u> ^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	90	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; Ogasawara et al., 1983, rrnJ; this study, rrnH; Wawrousek and Hansen, 1983, rrnD; E. Jarvis, unpublished results, rrnB; Green and Bott, 1983.

c) rrnH has no P1 promoter.

d) E. coli sequences were taken from Jinks-Robertson and Nomura, 1987.

Table 12. Cotransduction Values for pWR112 Insertions.^a

Kit 1 (trpC2, purA16, cysA14)

<u>Strain</u>	<u>Operon</u>	<u>Cm^r</u> <u>Pur⁺</u>	<u>Cm^r</u> <u>Cys⁺</u>
SB25-74	rrnO	72	25
SB25-191	rrnO	69	31
SB25-41	rrnA	57	42
SB25-96	rrnW	16	48

Kit 2 (trpC2, purB33, aroI906, dal-1)

<u>Strain</u>	<u>Operon</u>	<u>Cm^r</u> <u>Pur⁺</u>	<u>Cm^r</u> <u>Aro⁺</u>
SB25-77	rrnE	31	0

Kit 3 (trpC2, glyB133, metC3, tre-12)

<u>Strain</u>	<u>Operon</u>	<u>Cm^r</u> <u>Gly⁺</u>
SB25-43	rrnD	23
SB25-80	rrnD	34

Kit 7 (trpC2, ald-1, aroG932, leuA8)

Kit 8 (trpC2, hisA1, thr-5)

<u>Strain</u>	<u>Operon</u>	<u>Cm^r</u> <u>Aro⁺</u>	<u>Cm^r</u> <u>Thr⁺</u>
SB25-100	rrnB	30	27

a) All values are percent cotransduction with PBS1.

Table 13. Beta-galactosidase Activity in Strains Containing Integrated lacZ Fusions.

<u>Strain</u>	<u>rrn</u> or <u>other</u>	<u>Growth</u> <u>Medium</u>	<u>Doubling</u> <u>Time</u> <u>(min.)</u>	<u>Beta-gal</u> <u>(Miller units)</u>	<u>VY/MMA</u> ^a
<u>Controls</u> ^b					
168T-87	rrnA	MM	133	2.6	1.2
		VY	42	3.0	
SB25/pFW2	-	MM	75	3.6	1.6
		VY	42	5.7	
SB25-92	leu	MM	96	28.6	1.3
		VY	36	35.8	
<u>pWR112 Insertions</u>					
SB25-42	rrnO	MM	102	43.6	2.1
		VY	36	92.2	
SB25-74	rrnO	MM	102	39.1	2.4
		VY	44	94.2	
SB25-41	rrnA	MM	90	27.6	3.1
		VY	37	85.1	
SB25-96	rrnW	MM	96	33.3	2.9
		VY	40	96.5	
SB25-77	rrnE	MM	102	37.3	2.0
		VY	40	74.0	
SB25-43	rrnD	MM	105	15.4	2.2
		VY	32	34.5	
SB25-80	rrnD	MM	102	11.8	4.1
		VY	40	47.9	
SB25-100	rrnB	MM	110	17.7	3.0
		VY	45	52.7	

a) Ratio of Miller units of culture grown in VY versus minimal medium.

b) Control strain 168T-87 has a pGR102 insert in rrnA (no lacZ); SB25/pFW2 contains a replicating derivative of pCED6 (promoterless lacZ with a T7 terminator upstream); SB25-92 is an insertion in the leucine operon (see text).

Table 14. Sporulation Parameters of lacZ Insertion
Strains and Controls

<u>Strain</u>	<u>Operon</u>	<u>Viable cells/ml^a</u>	<u>Peak alkaline Phosphatase^b</u>
SB25	--	2.9 x10 ⁸	0.36
SB25-92	leu	4.1 x10 ⁸	0.92
SB25-42	rrnO	5.5 x10 ⁸	1.1
SB25-191	rrnO	6.6 x10 ⁸	1.1
SB25-41	rrnA	2.7 x10 ⁸	0.75
SB25-96	rrnW	2.6 x10 ⁸	0.74
SB25-77	rrnE	3.0 x10 ⁸	0.45
SB25-43	rrnD	3.5 x10 ⁸	0.41
SB25-80	rrnD	2.2 x10 ⁸	0.55
SB25-100	rrnB	3.2 x10 ⁸	0.50

a) Number of viable cells/ml after 15min. heating at 80°C determined after overnight growth in SG medium.

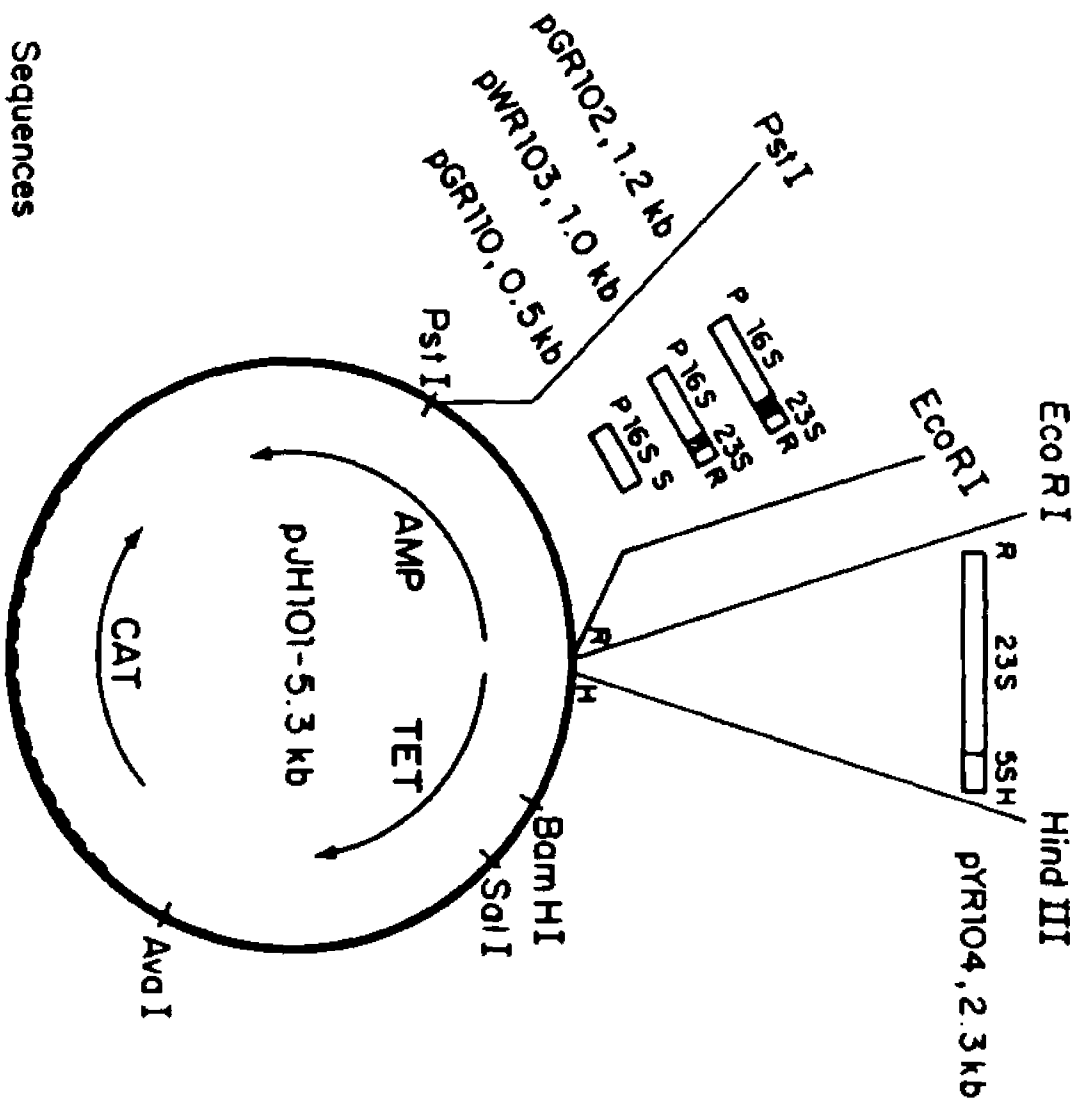
b) Alkaline phosphatase peak determined by taking hourly measurements beginning at the end of log phase. Units are absorbance of paranitrophenol at 410nm. The peak is at 5hrs. post-exponential.

Figure 1. Integrative Plasmids used in B. subtilis.

a) Integrative plasmids based on pJH101 (Ferrari et al., 1983) used for genetic mapping of rRNA operons (LaFauci et al., 1986). The construction of pGR102 and pWR103 was described in LaFauci et al., 1986; plasmids pGR110 and pYR104 are described in Jarvis et al., in press. The PstI site of pWR103 was lost during construction.

b) Generalized restriction map of a B. subtilis rRNA operon as proposed by Stewart et al., 1982.

d.



Sequences
derived from :

- E. coli
- - - S. aureus
- B. subtilis

b.

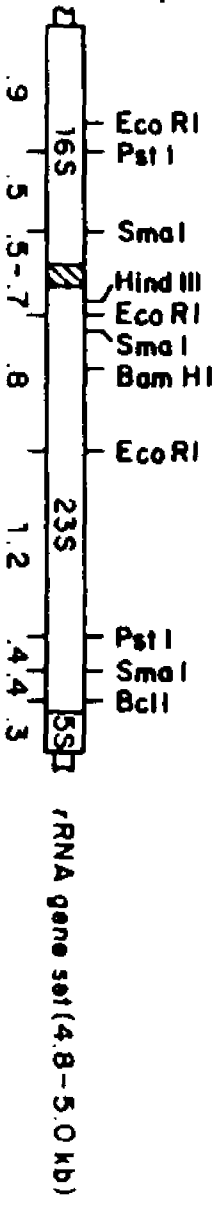


Figure 2. Construction of Integrative LacZ Fusion Plasmid pWR112.

The PstI-HindIII fragment of pGR102 (Figure 1a), containing 16S, tRNA, and 23S sequences was cloned into the PstI-HindIII sites of pDEB-1 (Schreier and Sonenshein, 1986), replacing most of the ampicillin resistance determinant, yielding pWR112 which confers chloramphenicol resistance to E. coli and to B. subtilis if it inserts in the chromosome. Restriction sites are: P; PstI, R; EcoRI, H; HindIII.

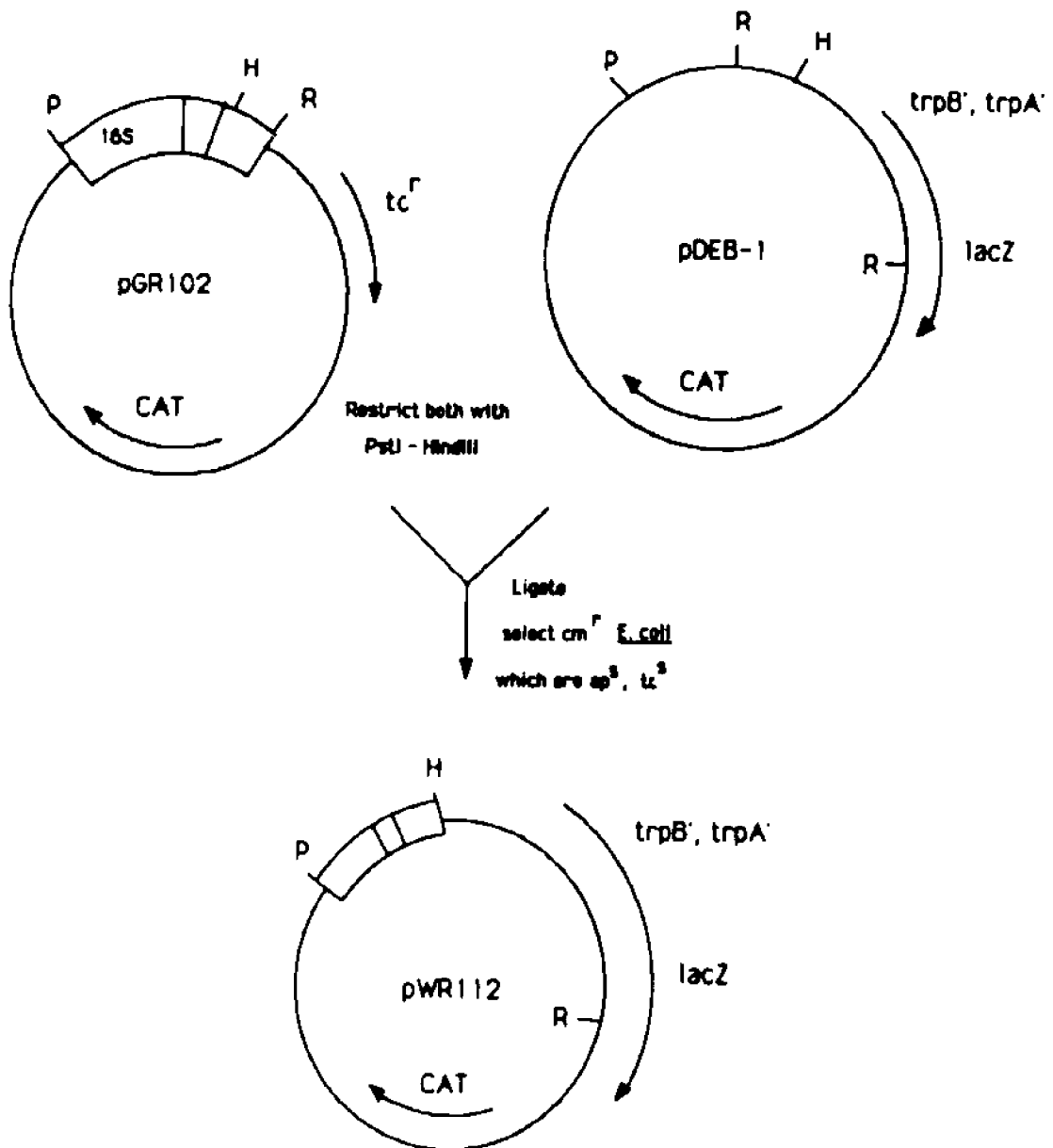


Figure 3. Construction of rrnJ Promoter Bearing Plasmid pWR113.

A 1.7Kb. PstI fragment from pGR151 (see text) was cloned between the two PstI sites of promoter search vector pKK232-8 (Brosius, 1984a), replacing the Ap^r gene of the cloning vector. The resulting plasmid (pWR113) gives resistance to at least 50 ug Cm/ml in E. coli. Restriction sites are: P; PstI, R; EcoRI, B; BamHI, Sm; SmaI, S; SalI, H; HindIII.

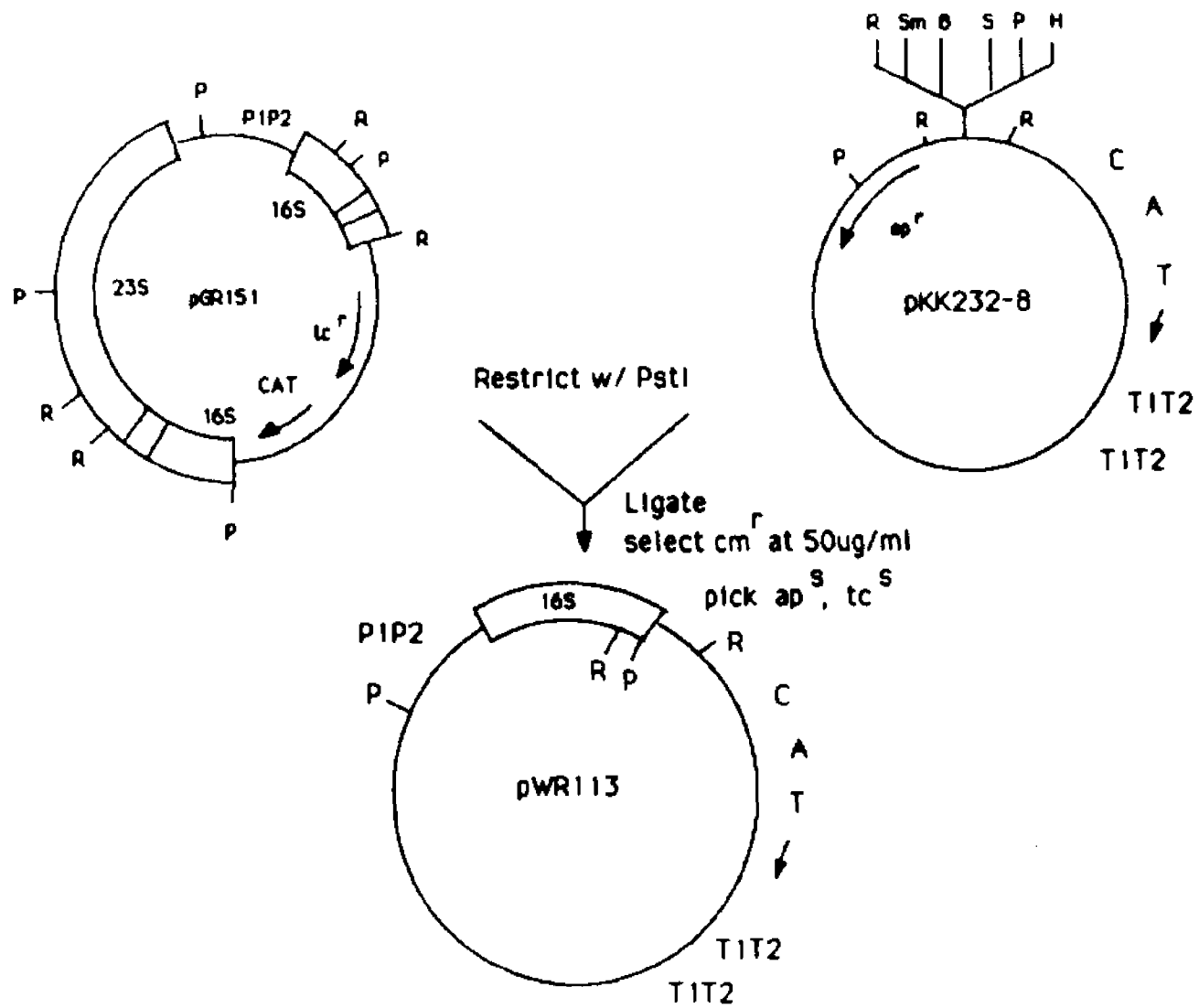


Figure 4. Strategy for Sequencing the Promoters of B. subtilis Operon rrnJ.

The 1.7Kb. PstI fragment was cloned into M13mpl8 and partially sequenced with the M13 universal 17mer sequencing primer (see Materials and Methods) to confirm the identity and orientation of the fragment. The arrows indicate sequence data obtained with the primer used. The HincII restriction site was identified by sequencing from the beginning of the 16S rDNA using a synthetic oligonucleotide primer (see Materials and Methods). The two PstI-HincII fragments were subsequently cloned and sequenced using the M13 17mer sequencing primer.

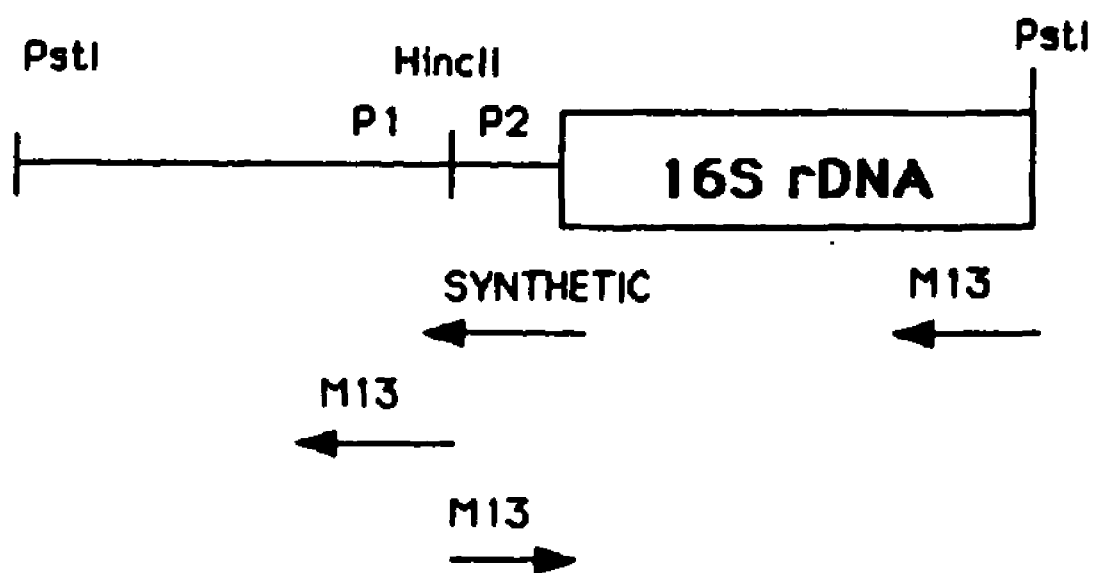


Figure 5. Insertions of Integrable Plasmids into Kit 1.

Autoradiograms of BclI digests of DNAs from insertions which map genetically in kit 1. DNAs restricted with BclI were electrophoresed through 0.75% agarose for 5-7 days at 15-20 mA, blotted and probed with an EcoRI-PstI 23S rDNA fragment isolated from pYR104 (Figure 1a). Each rRNA gene set near the origin has been inserted into except for rrnI, tentatively assigned to the 6.0Kb. homolog.

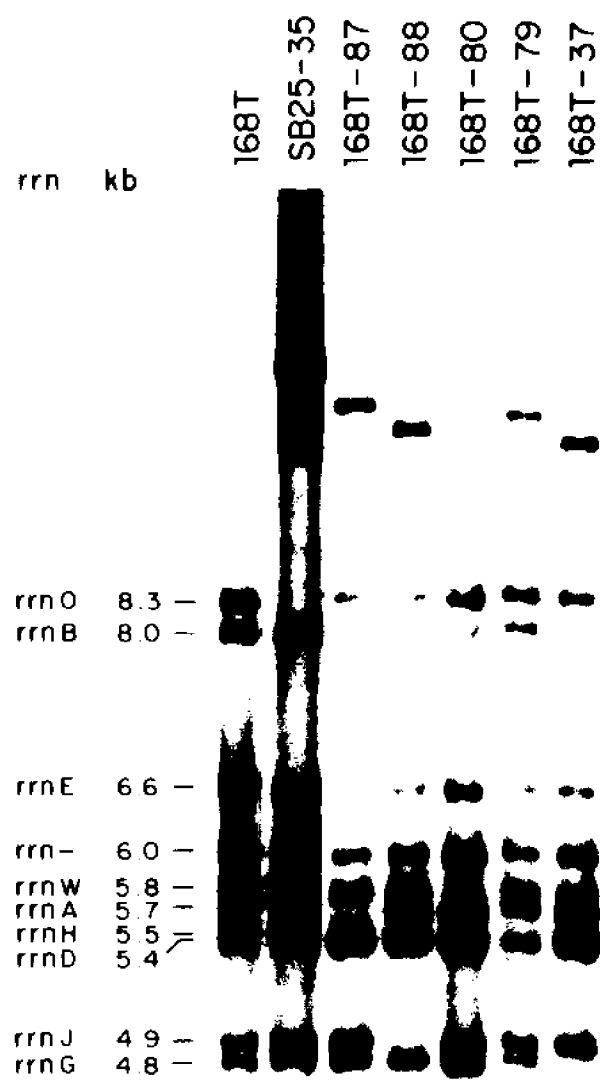


Figure 6. Genetic Map of B. subtilis rRNA Operons.

The genetic map of B. subtilis with the position of all 10 rRNA gene sets. The basic reference for the known loci is taken from the mapping kit strains of Dedonder et al., 1977, which are designated 1-9. Detailed transduction data for operons in kit 1 is presented in this study and in Jarvis et. al., in press.

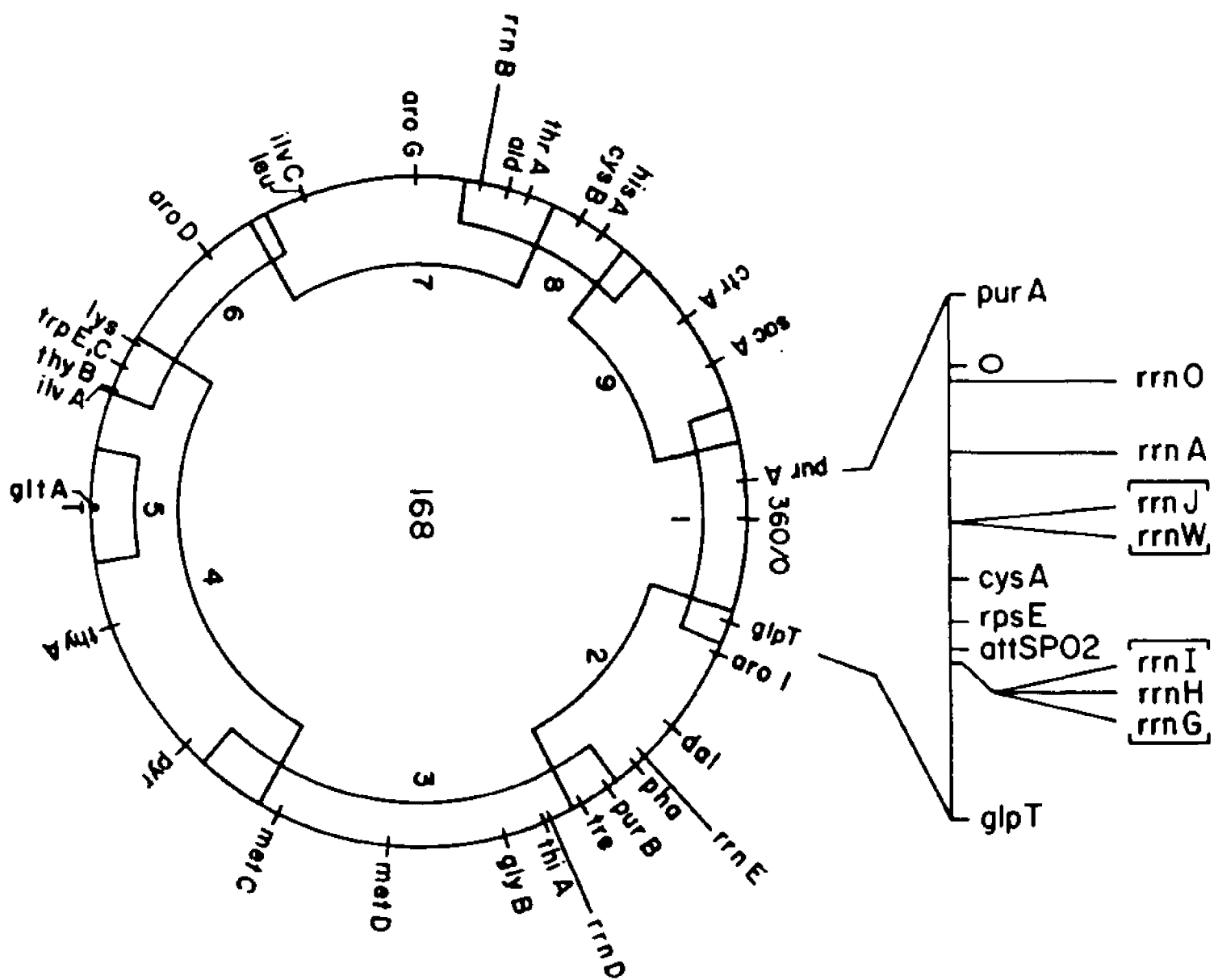


Figure 7. Southern Analyses of Strains with Deleted rRNA Genes.

a) Autoradiogram of BclI digested DNAs run on 0.75% agarose gels for 5-7 days at 15-20 mA, blotted and probed with 23S rDNA sequences from pYR104 (Figure 1a).

b) Autoradiogram of EcoRI digested DNAs run on 0.8% agarose gels for 48 hrs. at 15-20 mA, blotted and probed with pYR104.

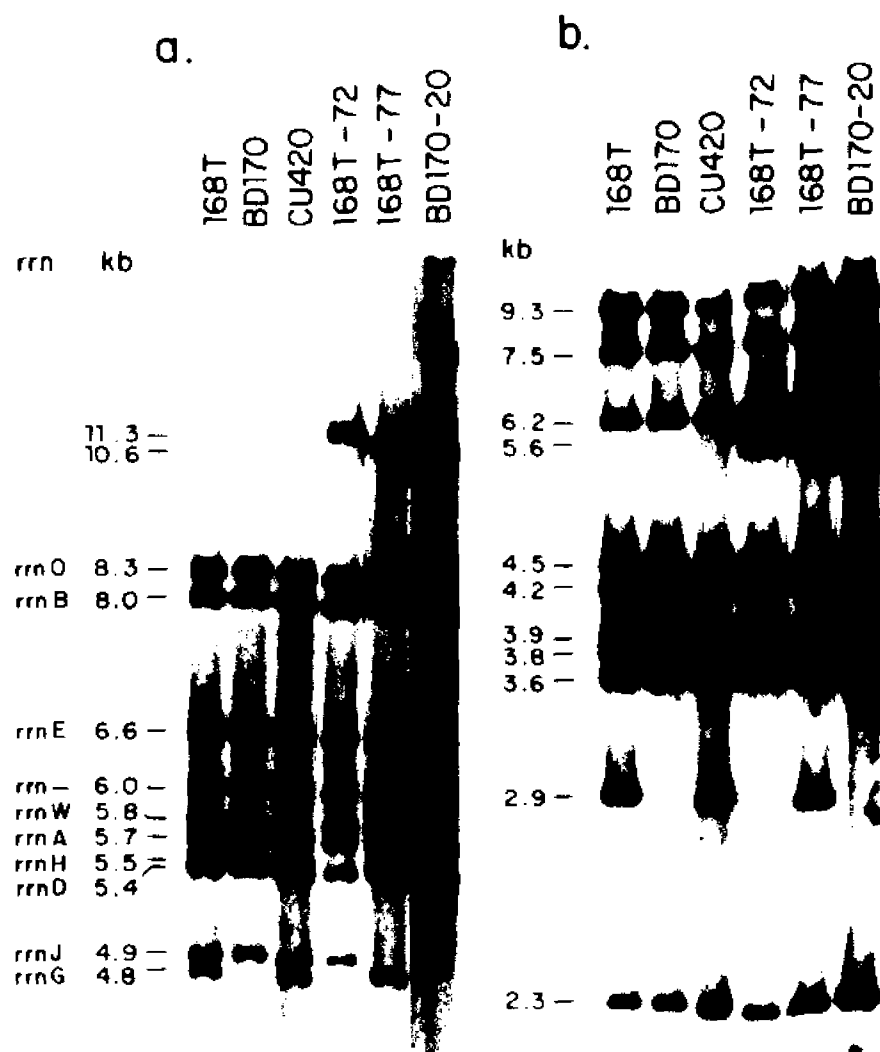


Figure 8. Model of Deletion of rDNA Sequences by Plasmid Insertion.

A prototype integrable plasmid containing PstI-EcoRI homologous rDNA sequences is shown inserting into the rrnH-rrnG tandem with concomitant deletion of the 23S-5S rDNA of rrnH and the beginning of the 16S rDNA of rrnG. A multimeric form of the plasmid (either circular or linear) interacts with the two closely situated rRNA gene sets by a double crossover between the repeated rrn sequences of the plasmid and the homologous regions on the chromosome. Restriction sites are as follows: B; BclI, R; EcoRI, P; PstI.

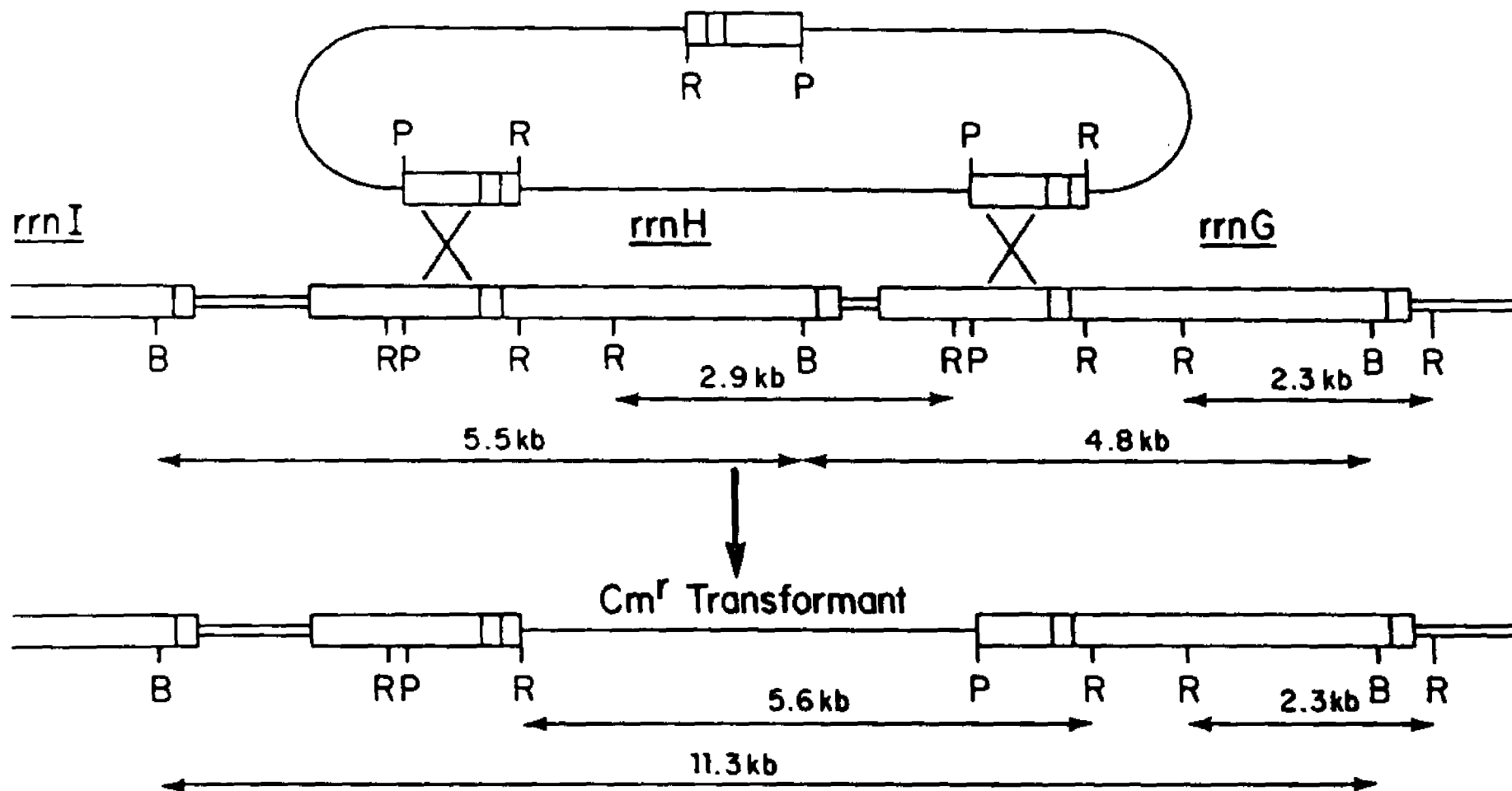
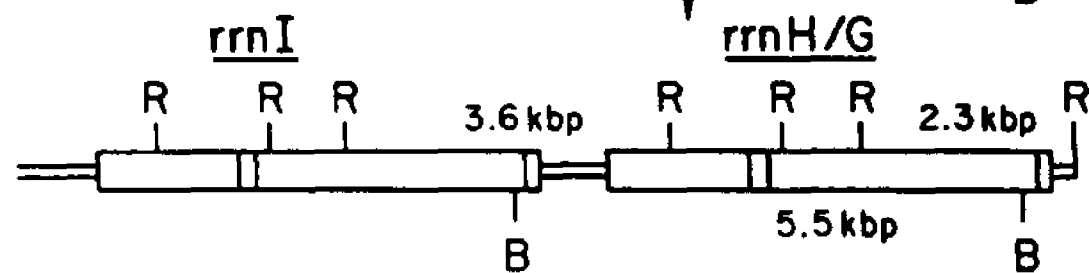
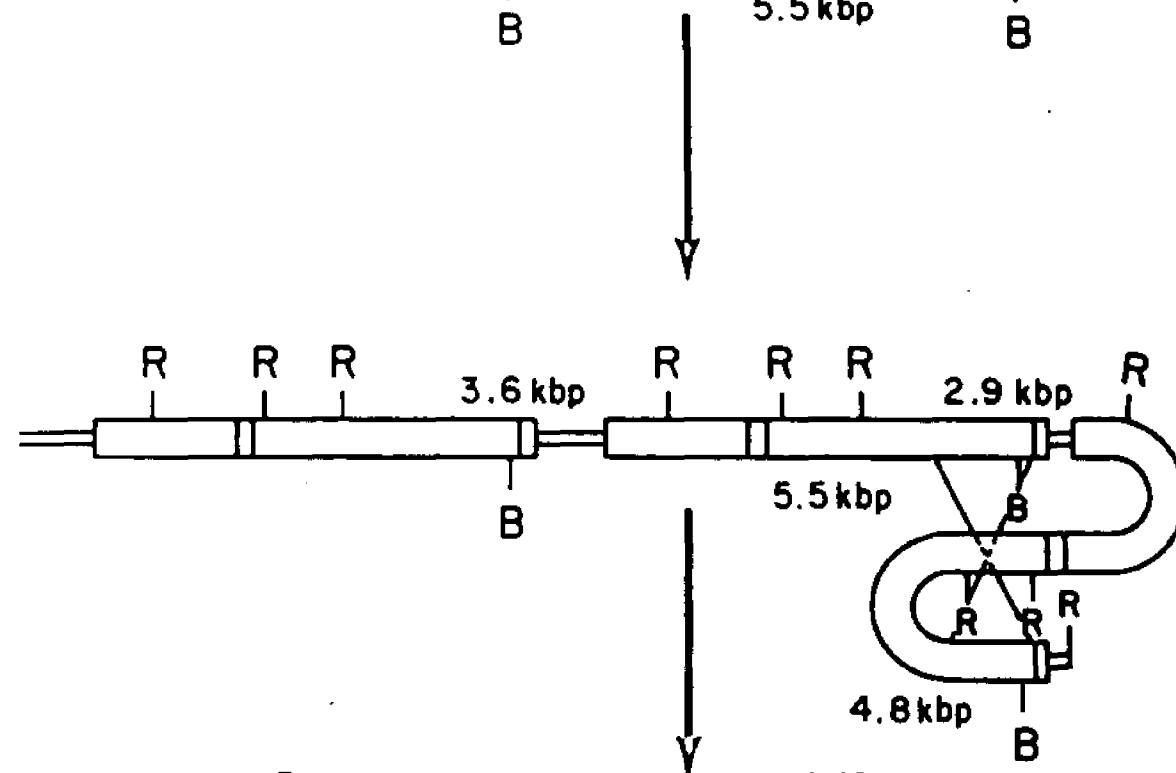
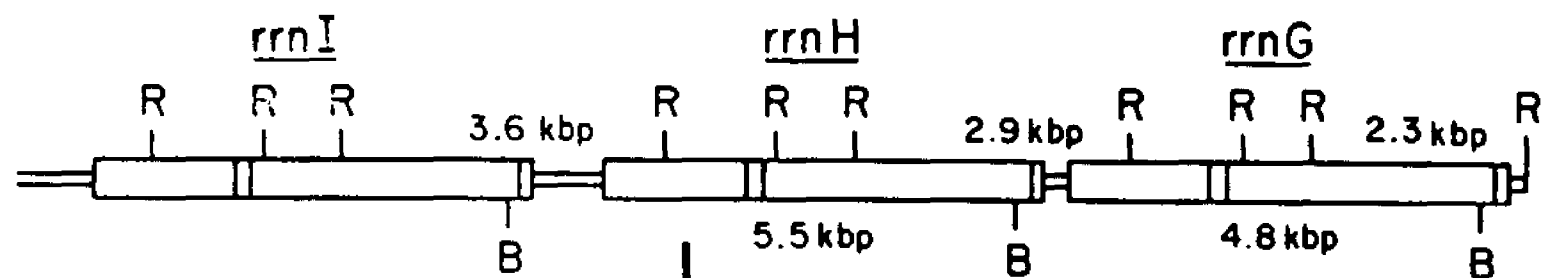
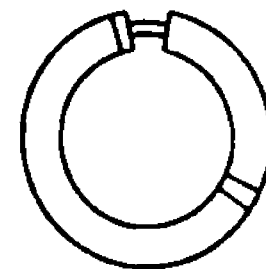


Figure 9. Model for Spontaneous Deletion of rRNA Operons.

Crossing over occurs between rrnH and rrnG, deleting an rRNA gene set equivalent, the intergenic spacer, and creating a "fusion" operon (rrnH/G) made up of the 5' part of rrnH and the 3' end of rrnG. DNA which is looped out is presumably lost. Restriction sites are as follows: B; BclI, R; EcoRI.



+



120

Figure 10. Southern Analysis of Rescued Plasmids pWR291 and pWR306.

a) Autoradiogram of EcoRI digested DNAs electrophoresed through 0.8% agarose, blotted, and probed with a 1.9Kb. EcoRI fragment from pGS227 (Stewart and Bott, 1983), containing the 5' end of the 16S gene.

b) Same as panel a except probed with a 23S 1.2Kb EcoRI-PstI fragment from pYR104.

c) Structures of the rrnJ,W doublet and rrnI,H,G triplet where deletions take place. R= EcoRI restriction site.

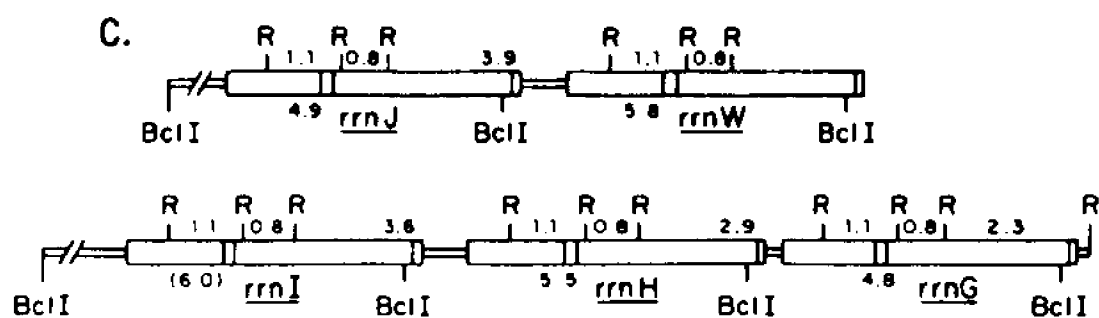
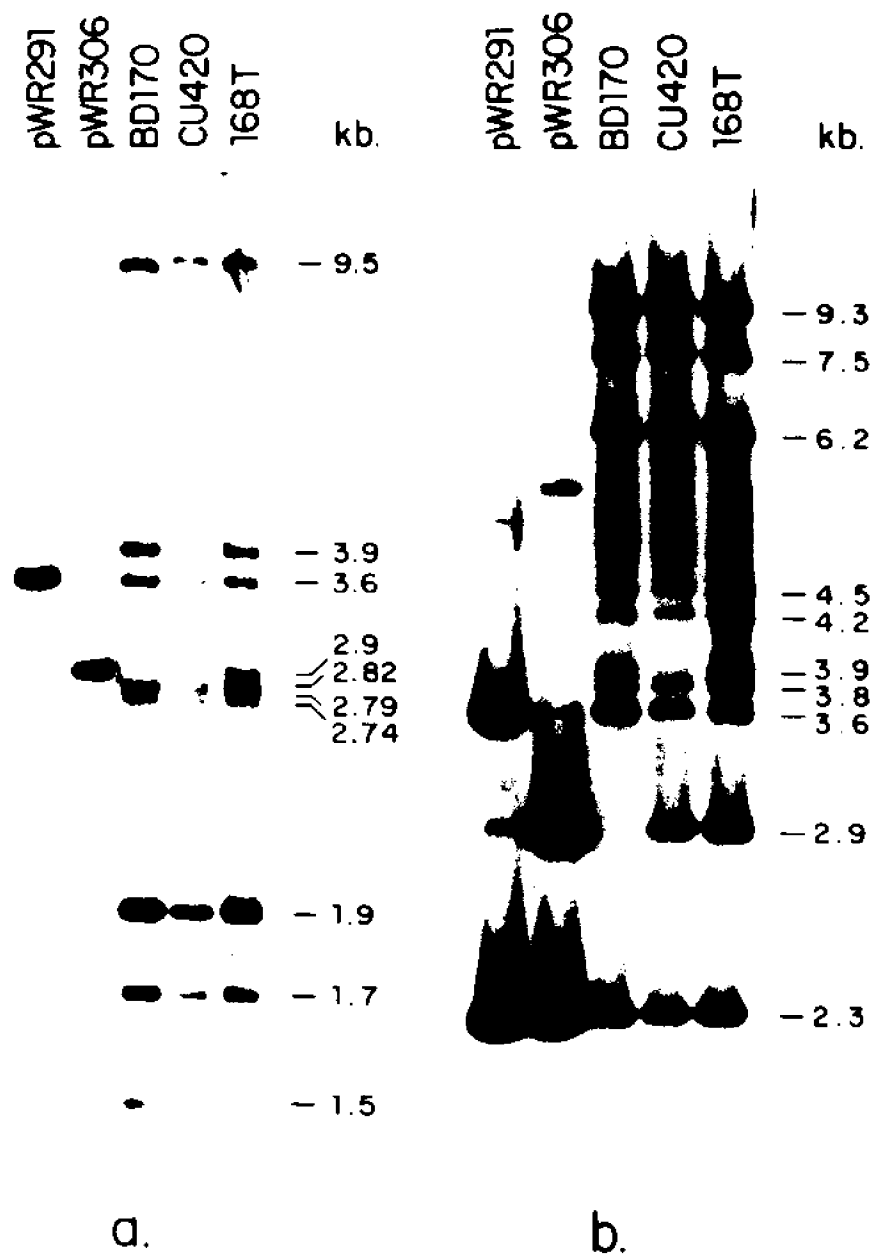


Figure 11. Spontaneous Deletion of rrnG During Insertion into Another Chromosomal Location.

a) Autoradiogram of BclI restricted DNAs which were separated on 0.75% agarose gels, blotted and probed with a 23S rDNA fragment. The DNA loaded is as follows:

1. Lambda phage DNA (HindIII restricted).
2. SB25-40
3. SB25-39

The control strain SB25-40 has an insertion of pGR110 into rrnG, so the 4.8Kb. homolog is displaced (bottom arrow). In strain SB25-39, the same 4.8Kb. homolog is missing, but the 5.4Kb. homolog (top arrow) is displaced, giving a high molecular weight band larger than strain SB25-40. The sizes of lambda fragments in Kb. are shown at the left.

Figure 11. continued.

b) Autoradiogram of EcoRI restricted DNAs electrophoresed through 0.8% agarose, blotted and probed with pYR104.

1. Lambda phage DNA (HindIII digested)
2. SB25
3. SB25-39
4. SB25-40

Strain SB25 and SB25-40 both show 10 EcoRI fragments as expected if no deletions have taken place. SB25-39 is missing the 2.9Kb. homolog (bottom arrow), associated with rrnG deletion. The top arrow indicates vector sequences released by EcoRI digestion.

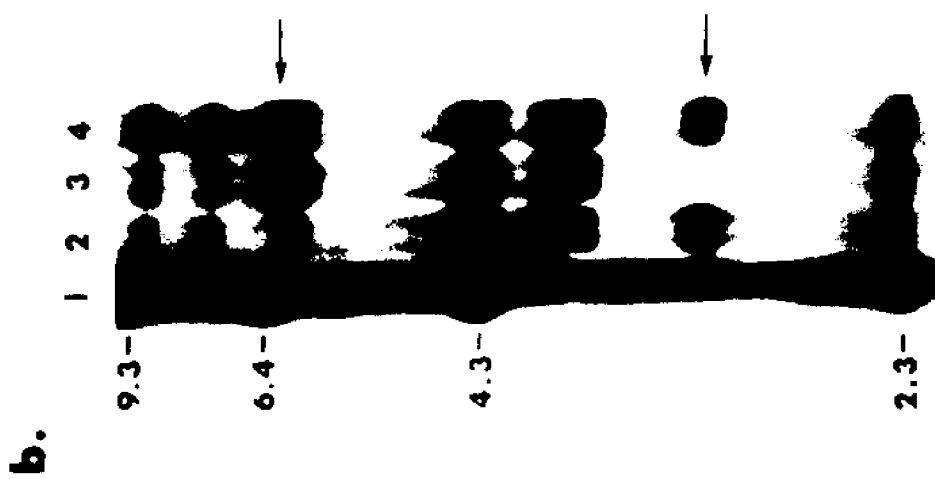
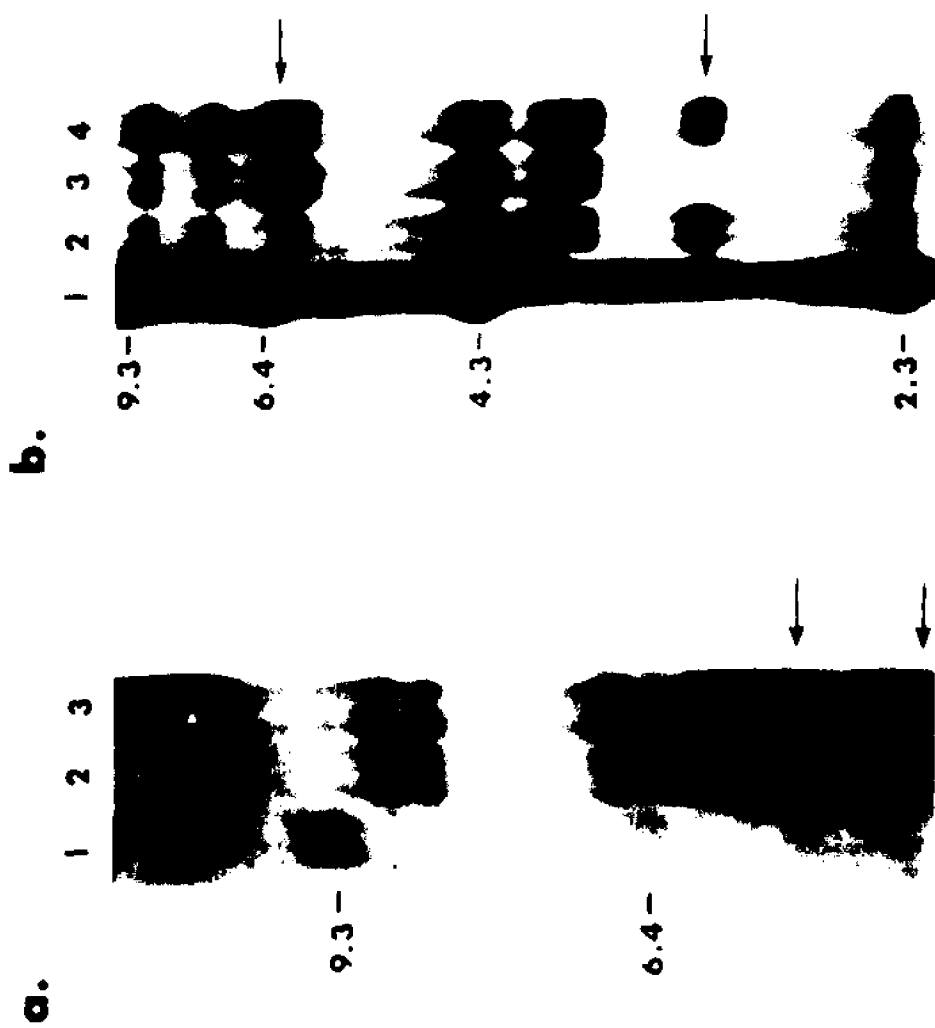


Figure 12. Model of Integration and Rescue of Mapping Plasmid pGR102.

The insertion of pGR102, pWR103, or pGR110, by a Campbell-like event leads to the entire plasmid integrated into the chromosome with the cloned region of homology duplicated. Upon BclI restriction (bottom line) and recircularization, sequences 5' to the 16S rDNA are rescued, up to the BclI site in the chromosomal DNA. The arrow indicates a small HindIII fragment (95bp.) which is not seen in restriction analysis.

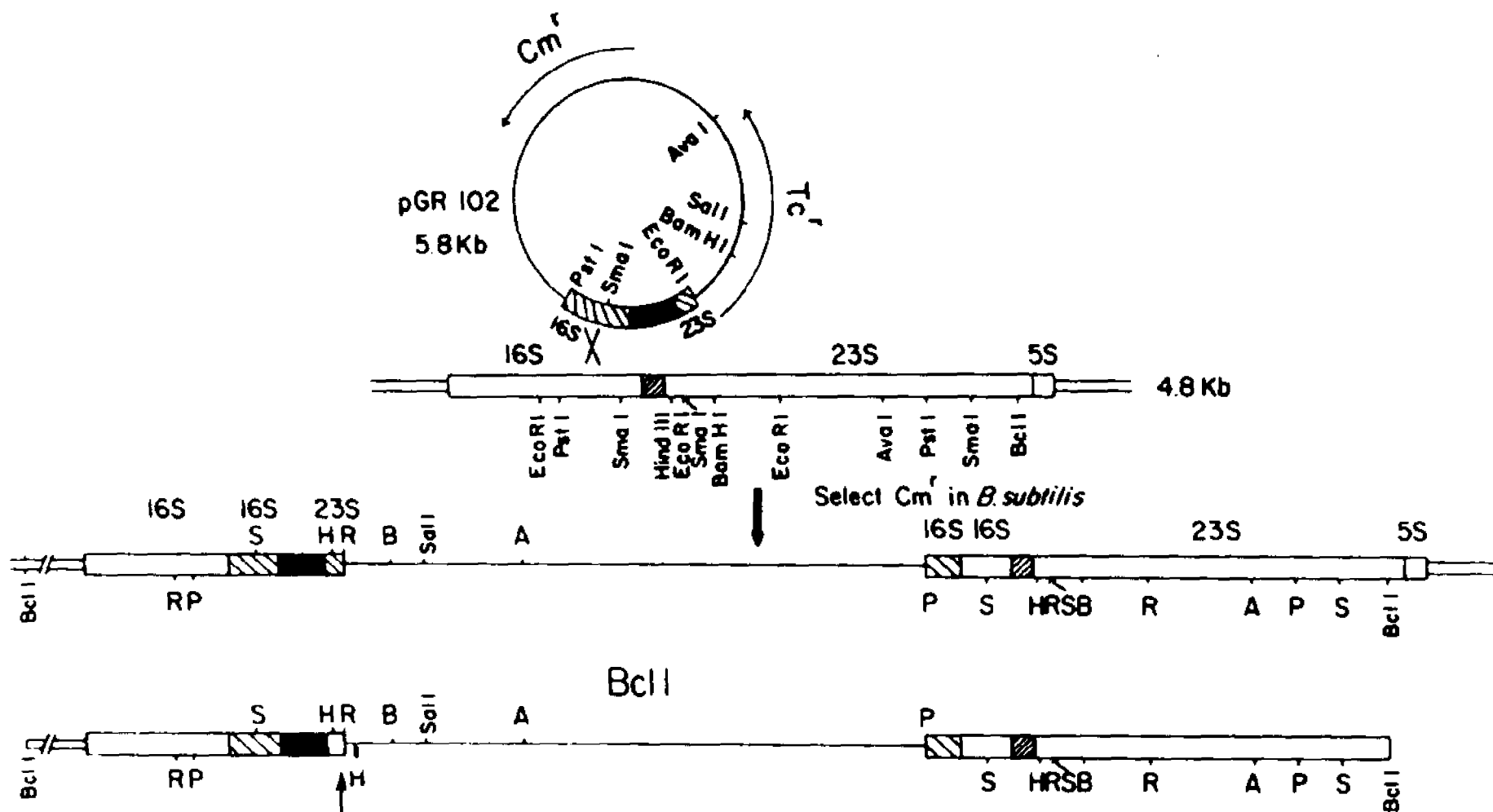


Figure 13. Colony Filter Hybridization.

A sample autoradiogram of a filter hybridization is shown. The E. coli colonies are numbered from 1 to 25, increasing from left to right. The control or possible rescue plasmids carried by the colonies are as follows:

1 and 2. pJH101

3 and 4. pWR182

5 through 12. Rescues from B. subtilis strain BD79-175.

13 through 25. Rescues from B. subtilis strain BD170-143.

E. coli colonies carrying potential rescues and controls were replica plated from a grid onto nitrocellulose and treated as described in Materials and Methods. The probe used was the EcoRI-HindIII fragment of pYR104 (Figure 1a).



Figure 14. Model of Spontaneous Deletion of Rescued Sequences.

The instability of rescued plasmids containing 5' regulatory regions leads to deletion of all rescued DNA, leaving the original insertional vector. The model predicts that a crossover event occurs during growth in E. coli where the directly repeated elements of the rescue plasmid recombine to eliminate any rescued sequences. A typical rescue plasmid is shown. Restriction sites are: P; PstI, R; EcoRI.

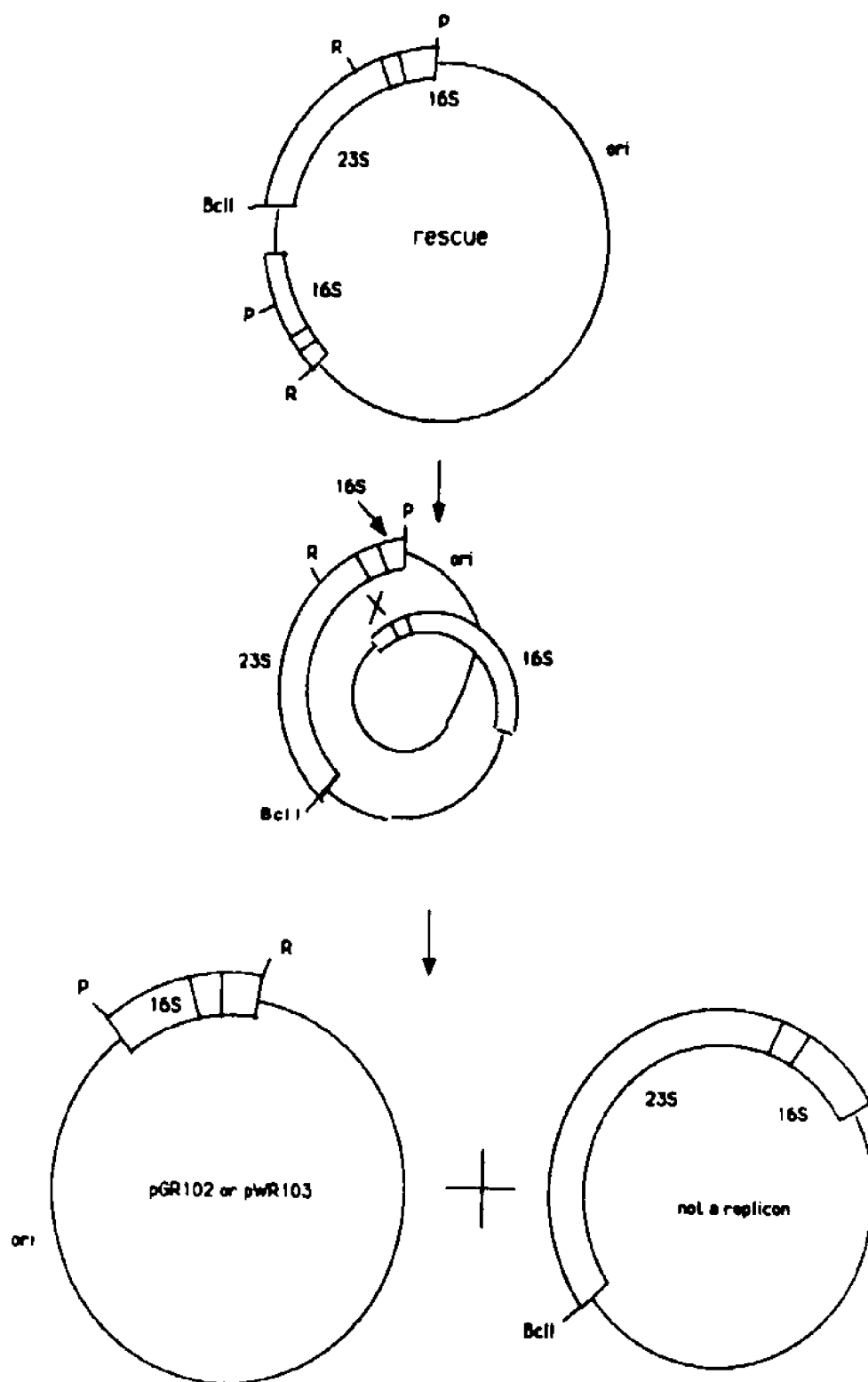


Figure 15. Analysis of pWR179 rrnO Rescue Plasmid.

a) Restriction fragment map of pWR179. The 5' end of the rescue was expected to contain the gyrA and part of the gyrB gene, based on Lampe and Bott, 1985, and Moriya et al., 1985. Restriction sites are: P; PstI, R; EcoRI, B; BamHI.

b) Autoradiogram of a Southern blot of BamHI-EcoRI restricted DNAs run on a 0.8% agarose gel at 20mA overnight and probed with a 2.2Kb. BamHI-EcoRI fragment from plasmid pAK1 (Lampe and Bott, 1984) containing the gyrA gene.

1. pAK1
2. pWR179.
3. 168T

The arrow indicates the 2.2Kb. BamHI-EcoRI fragment used as the probe. The fragment hybridizes to an identical fragment from pWR179 and in 168T chromosomal DNA. The additional fragments seen in lane 3 are a result of incomplete BamHI digestion.

Figure 2b shows a schematic diagram of a three-dimensional structure, likely a protein complex. The structure is composed of several interconnected components. Labels 1, 2, and 3 are placed near different parts of the structure. An arrow points to a specific feature on the right side of the structure.

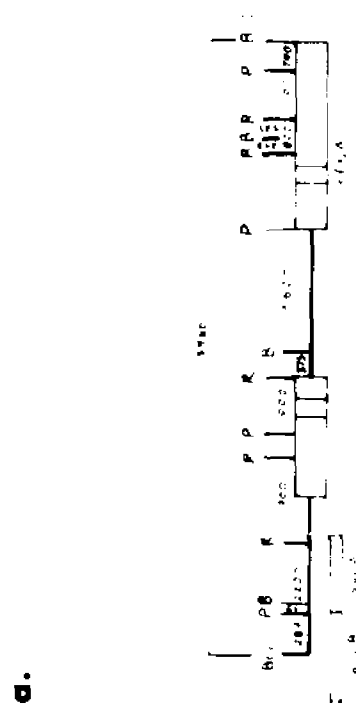


Figure 16. Restriction Analysis of pWR113 Carrying the Promoters of B. subtilis Operon rrnJ.

Restriction analysis of pWR113 and its parent plasmids is shown on a 0.8% agarose mini-gel run at 75mA for 1hr. in the presence of ethidium bromide. The following DNAs were restricted and loaded:

1. Lambda phage DNA; HindIII restricted.
2. pKK232-8; PstI restricted.
3. pWR113; PstI restricted.
4. pGR151; PstI restricted.
5. pWR113; EcoRI restricted.

The upper arrow indicates the 1.7Kb. PstI promoter bearing fragment of pGR151 (lane 4) is present in pWR113 (lane 3), replacing the smaller PstI fragment of pKK232-8. The lower arrow identifies the 400bp. EcoRI fragment released from pWR113, confirming that the PstI fragment is cloned in the direction where the promoters can transcribe the CAT gene of pKK232-8 (see text and Figure 3).

1 2 3 4 5

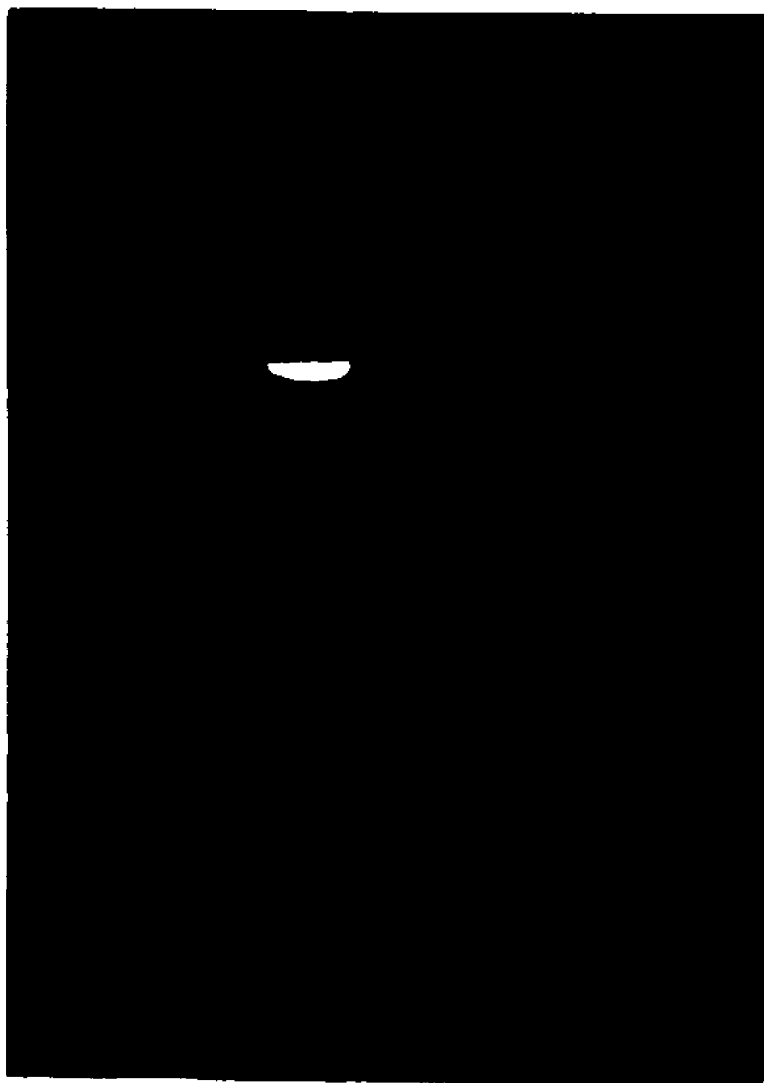


Figure 17. DNA Sequence of the 16S rRNA Gene of rrnJ.

The nucleotide sequence beginning from the PstI site found in the 16S rRNA determinant of rrnJ (top line) is shown compared to the same location in rrnB (lower line; Green et al., 1985). The sequences are identical. The last 11 nucleotides of the rrnJ sequence are from the M13 polylinker region.

```

      1  AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGA  50
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1219  AAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGA 1268

      51  GCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT  100
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1269  GCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCT 1318

      101  AAGTGTTAGGGGGGTTTCCGCCCCTTAGTGCTGCAG...GCAT...GCA  142
      | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
1319  AAGTGTTAGGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTGAGCA 1368

      143  AGCT  146
           | |
1369  ..CT  1370

```

Figure 18. DNA Sequence of the B. subtilis rrnJ Promoter Region.

The 380 bases of DNA sequence obtained from the PstI fragment of pWR113 (see Figure 4) are shown. The 16S structural gene begins at base 362 (boldface), based on comparison to published B. subtilis sequences. Two putative promoters can be found, each with -35 and -10 consensus boxes (in italics) separated by 17bp. Homology to Box A sequences is underlined (see text). The sense strand is shown from 5' to 3'.

1 CCCCTTCTAT TCGCGATGTG CTGTTATACC CGCAAAGAGA CAACGCTAAT
 51 AAAAAAGAGC GGTATCCTCC ATAGGGAAAG GATGCCGCTT CATT TTTAAA
 101 TCCAGTAGT ATTTCTTCAA AAAA ACTATT ⁻³⁵ GCACTATTAT ^{P1} TTACTAGGTG
 151 GTATATTATT ATTCGTTGCC GCTAAACAAG GCGATAACGA AAAAAAGAAC
 201 TTCAAAAAAA GTTATTGACT ⁻¹⁰ TCACTGAGTC ⁻³⁵ AACGAGTTAT ^{P2} AATAATAAAG
 251 TCGCTTGAAC GAAGTGAACA AAATGATCTT TGAAAACTAA ⁻¹⁰ ACAAGACAAA
 301 ACGTACCTGT TAATTCAGTT TTTAAAAATC GCACAGCGAT GTGCGTAGTC
 351 AGTCAA ACTA ¹⁶⁸ CTTTATCGGA GAGTTTGATC

Figure 19. Analysis of Open Reading Frame Upstream of rrnJ.

A possible mRNA followed by a large stem-loop structure can be made from the sequences upstream of the putative dual promoters. An open reading frame coding for at least 15 amino acids is followed by two stop codons. An RNA with the same sense as the DNA in Figure 18 could form a stem-loop with a free energy of -20.0 Kcal/mole, having a loop of 10 bases and a stem with 36 paired residues and three bulged nucleotides. The putative P1 promoter of rrnJ would be found 22 bases away from this possible transcription terminator.

Figure 20. Secondary Structure of rrnJ Upstream Region.

A larger secondary structure can be formed with the DNA upstream of the rrnJ P1 promoter, containing one large and one small stem-loop. This structure would involve bases 13 to 117 of the sequence shown in Figure 18. An RNA with the same sense as the DNA in Figure 18 could form this structure with a free energy of -28.4 Kcal/mole. The same structure might form in the DNA, with the exception of the 4 GU pairs indicated.

Figure 21. Upstream Regions of B. subtilis rRNA Gene Sets.

The six available DNA sequences from upstream of B. subtilis rRNA gene sets are aligned by their P2 and 16S sequences. The 16S rDNA begins with the three T residues as indicated. Gaps are introduced to better align the sequences. The P2 and P1 promoter sequences are boxed (see text for detailed analysis). The references for the sequences are: rrnO,A; Ogasawara et al., 1983, rrnB; Stewart and Bott, 1983, rrnH; Wawrousek and Hansen, 1983, rrnD; E.D. Jarvis, unpublished results, rrnJ; this study.

10 20 30
 rrnJ A A A A A A C T A T T G C A C T A T T A T T A C T A G G
 rrnA T T A A T T G A T A T T A T G T A T T G A C T T A G A C A A
 rrnO T T T A C A G T C A T A A A A A T T A T G G T A T A A T C A
 rrnB A C T A T T G C A A T A A A T A A A T A C A G G T G T T A T
 rrnD T T G A C T C T G A T T C T T G A C C G T G T T A T A T T A

P1

40 50 60
 rrnJ T G G T A T A T T A T T A T T C G T T G C C G C T A A A C A
 rrnA C T G A A G G T G T T A T T C T A A T A T C G C T G A T G A
 rrnO T T T C T G T T G T C T T T T A A A G A C A C A A G C A T
 rrnB A T T A T T A A A C G T C G C T G A T G C A C A G C G G A C
 rrnD T T A A A C G T C C T G A T G C G C T T C T G A G A A A A C

70 80 90
 rrnJ A G G C G A T A A C G A A A A A A A G A A C T T C A A A A A
 rrnA C G A A C A G C T T T T T T G A A A A G A A A A T G C T A A A
 rrnO G A C C A T T A T G A C T A G T A A A A A A C T T T T T C A A
 rrnB A C A A C T A G A T G C T T C A A A A A C A A C T T G A A A A
 rrnD A A G C T C A C A G C G G C G G A A A A T A A A T C A A A
 rrnH six tRNAs --- A A A G T T T T A A A A

100 110 120
 rrnJ A A G T T A T T G A C T T C A C T G A G T C A A G G A G T
 rrnA A A G T T G T T G A C A G T A G C G G C G G T A A A T G T
 rrnO A A A A G T A T T G A C C T A G T T A A C T A A A A T G T
 rrnB A A G T T G T T G A C A A A A A A G A A G C T G A A T G T
 rrnD A A A A C A T T T G A C A A A A G A A A G T C A A A T G T
 rrnH A A G T T A T T G A C T T T G A A G A A G T G A C A T T G

P2

130 140 150
 rrnJ T A T A A T A A T A A A G T C G C T T G A A A C G A A G T G A
 rrnA T A T G A T A A T A A A G T C G C T T A A A G G A G C G G T
 rrnO T A C T A T T A A G T A G T C G C T T T G A G A G A A G C
 rrnB T A T A T T A G T A A A G C T G C T T C A T G G A A G T A
 rrnD T A T A T T A A T A A A G T C G C T T C A A C A A G A A G
 rrnH T A T A C T A A T A A A G T T G C T T T A A C A A A G C G G

160 170 180
 rrnJ A C A A A A T G A T C T T T G A A A A C T A A A C A
 rrnA A A A C A A A G T T C T T T G A A A A C T A A A C A
 rrnO A C A C A A G T T C T T T G A A A A C T A A A C A
 rrnB A C G A A A T G A T C T T T G A A A A C T A A A C A
 rrnD T G A A A A T G A T C T T T G A A A A C T A A A C A
 rrnH A C A A A C A A A A T G A T C T T T G A A A A C T A A A C A

		190		200		210
<u>rrnJ</u>	A G A C A A A A C G	T A C C T G T T A A	T T C A G T T T T T			
<u>rrnA</u>	A G A C A A A A C G	T A C C T G T T A A	T T C A T T T T T T			
<u>rrnO</u>	A G A C A A A A C G	T A C C T G T T A A	T T C A T T T T T T			
<u>rrnB</u>	A G A C A A A A C G	T A C C T G T T A A	T T C A G T T T T T			
<u>rrnD</u>	A G A C A A A A C G	T A C C T G T T A A	T T C A G T T T T T			
<u>rrnH</u>	A G A C A A A A C G	T A C C T G T T A A	T T C A G T T T T T			

		220		230		240
<u>rrnJ</u>	A A A A A T C G C A	C A G C G A T G T G	C G T A G T C A G T			
<u>rrnA</u>	A T A A A T C G C A	C A G C A A T G T G	C G T A G T C A G T			
<u>rrnO</u>	A T A A A T C G C A	C A G C G A T G T G	C G T A G T C A G T			
<u>rrnB</u>	A A A A A T C G C A	C T G C G A T G T G	C G T A T C A T			
<u>rrnD</u>	A A A A A A C G C A	C A G C G A T G T G	C G T A G T C A G T			
<u>rrnH</u>	A A A A A T C G C A	C A G C G A T G T G	C G T A G T C A G T			

		250		260		270
<u>rrnJ</u>	C A A A C T A C					
<u>rrnA</u>	C A A A C T A C					
<u>rrnO</u>	C A A A C T A G G G	C C T G C A C G A C	G C A G G T C A C A			
<u>rrnB</u>	C A A A C T A G G G	C C T G C A C G A C	G C A G G T C A C A			
<u>rrnD</u>	C A A A C T A G G G	C C T G C A C G A C	G C A G G T C A C A			
<u>rrnH</u>	C A A A C T A C					

		280		290		300
<u>rrnJ</u>						
<u>rrnA</u>						
<u>rrnO</u>	C A G G T G T C G C	C G C A G G A T G C	G G T G A A C T T A			
<u>rrnB</u>	C A G G T G T C G C	C G C A G G A T G C	G G T G A A C T T A			
<u>rrnD</u>	C A G G T G T C G C	C G C A G G A T G C	G G T G A A C T T A			
<u>rrnH</u>						

		310		320		327
<u>rrnJ</u>						
<u>rrnA</u>						
<u>rrnO</u>	A C C T G T G A T C	C A	T T T A T C G G	A G A G T T T		
<u>rrnB</u>	A C C T G T G A T C	C A	T T T A T C G G	A G A G T T T		
<u>rrnD</u>	A C C T G T G A T C	C A	T T T A T C G G	A G A G T T T		
<u>rrnH</u>			T T T A T C G G	A G A G T T T		

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Figure 22. Comparison of Upstream Sequence Elements with Antitermination Sequences.

The Box A sequences implicated in antitermination in E. coli rRNA operons and in bacteriophage lambda are compared to sequence elements found in B. subtilis rRNA leaders and spacer regions. The small letters indicate that no consensus is available; approximately half of the sequences show one base, while the others have the alternate base. Sequences for the lambda and E. coli genes are taken from Morgan, 1986. The B. subtilis sequences are compiled from Ogasawara et al., 1983, Green et al., 1985, Wawrousek and Hansen, 1983, E.D. Jarvis, unpublished results, and this study.

Lambda nut _L	C	G	C	T	C	T	T	A	A	A	A	T	T
Lambda nut _R	C	G	C	T	C	T	T	A	C	A	C	A	T
<i>E. coli</i> leader	T	G	C	T	C	T	T	T	A	A	C	A	T
<i>E. coli</i> <u>16S-23S</u> spacer	T	G	C	T	C	T	T	T	A	A	A	A	T
<i>B. subtilis</i> leader	t/a	G	t/a	T	C	T	T	T	G	A	A	A	C
<i>B. subtilis</i> <u>16S-23S</u> spacer	t/a	G	T	T	C	T	T	T	G	A	A	A	C

Figure 23. CAT Specific Activities of pKK232-8 Derivatives in E. coli HB101.

CAT specific activity was determined by the method of Shaw, 1975, and lines calculated by linear regression. Slopes were calculated from the plots by measuring the change in CAT activity ($\times 10^{-3}$) divided by the change in growth rate.

a) Plot of CAT specific activity versus growth rate for E. coli HB101 carrying pKK351Ec. with the PlP2 promoters of ribosomal operon B of E. coli. The positive slope (slope = 2.15) indicates growth-rate regulation.

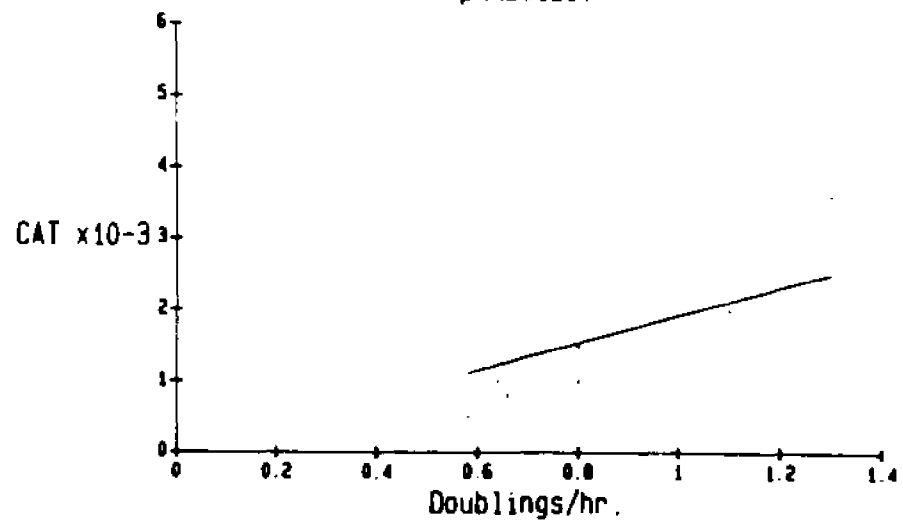
b) Same plot as above for cells carrying plasmid pKK292Ec. with the Pl promoter of E. coli rrnB. The slope = 7.0.

c) Same as above for cells carrying pKKtet with the tetracycline resistance gene promoter from pBR322. The slope is zero.

d) Identical plot for cells carrying pWR113 carrying the dual promoters of B. subtilis rrnJ. The slope is 7.3.

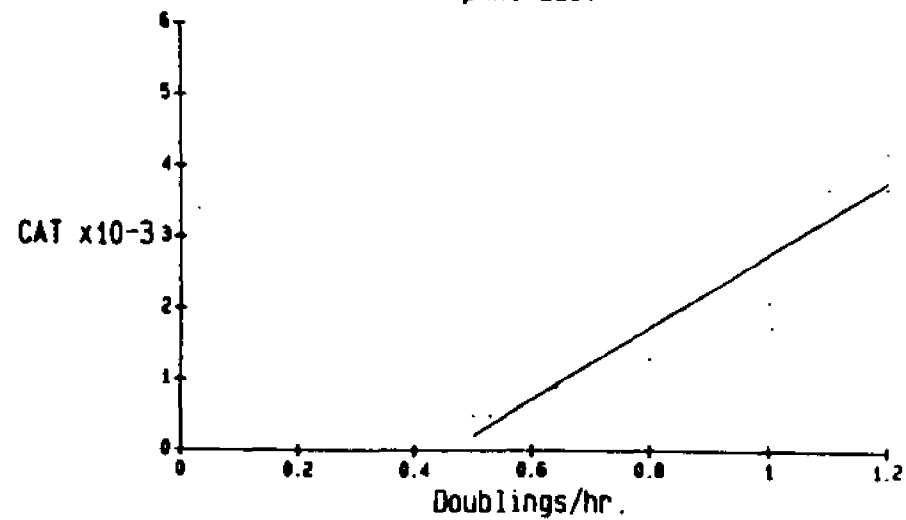
a.

pKK351Ec.



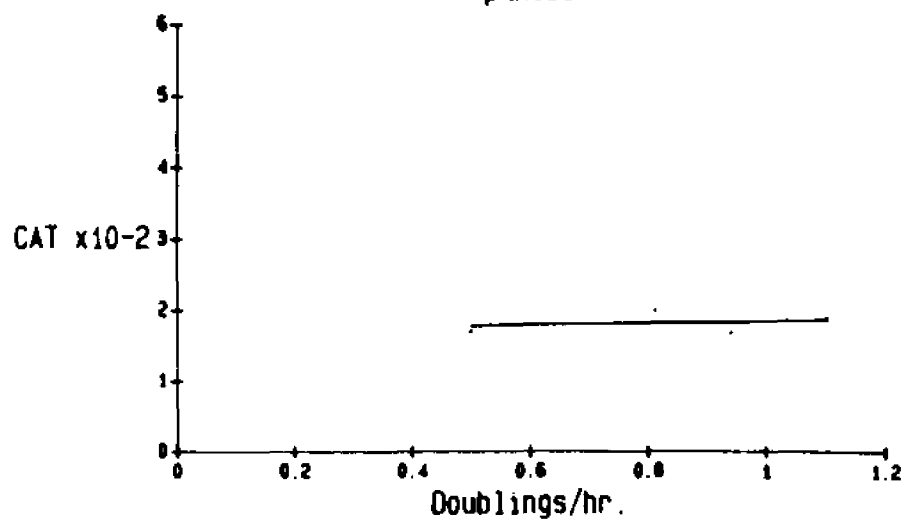
b.

pKK292Ec.



c.

pKKtet



d.

pWR113

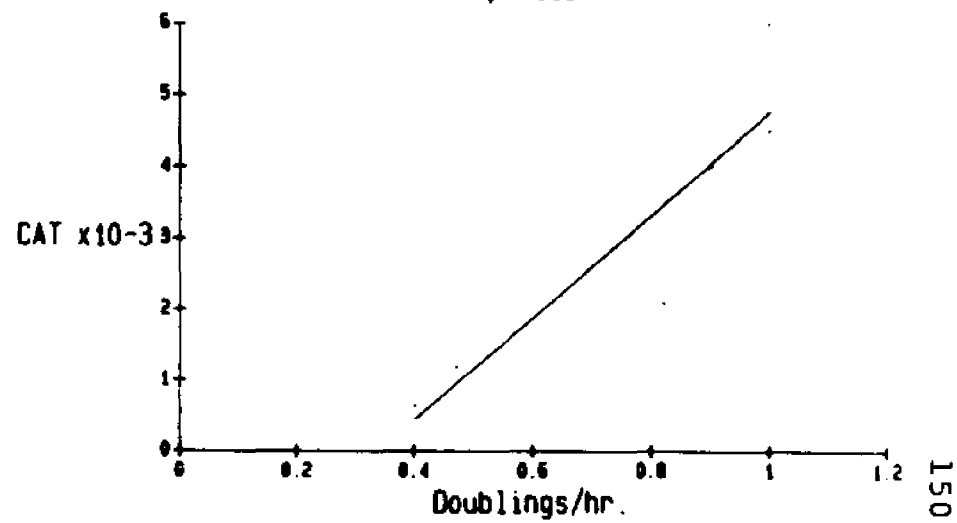


Figure 24. Integration of pWR112 into a B. subtilis rRNA Gene Set.

Integrable lacZ fusion plasmid pWR112 was used to transform B. subtilis SB25 to Cm^r. A Campbell-like model of integration predicts that the promoterless lacZ gene of the vector will be placed under the control of the ribosomal promoters resident in the chromosome at the specific site of insertion. Restriction endonuclease sites are as follows: P; PstI, H; HindIII, R; EcoRI, B; BclI.

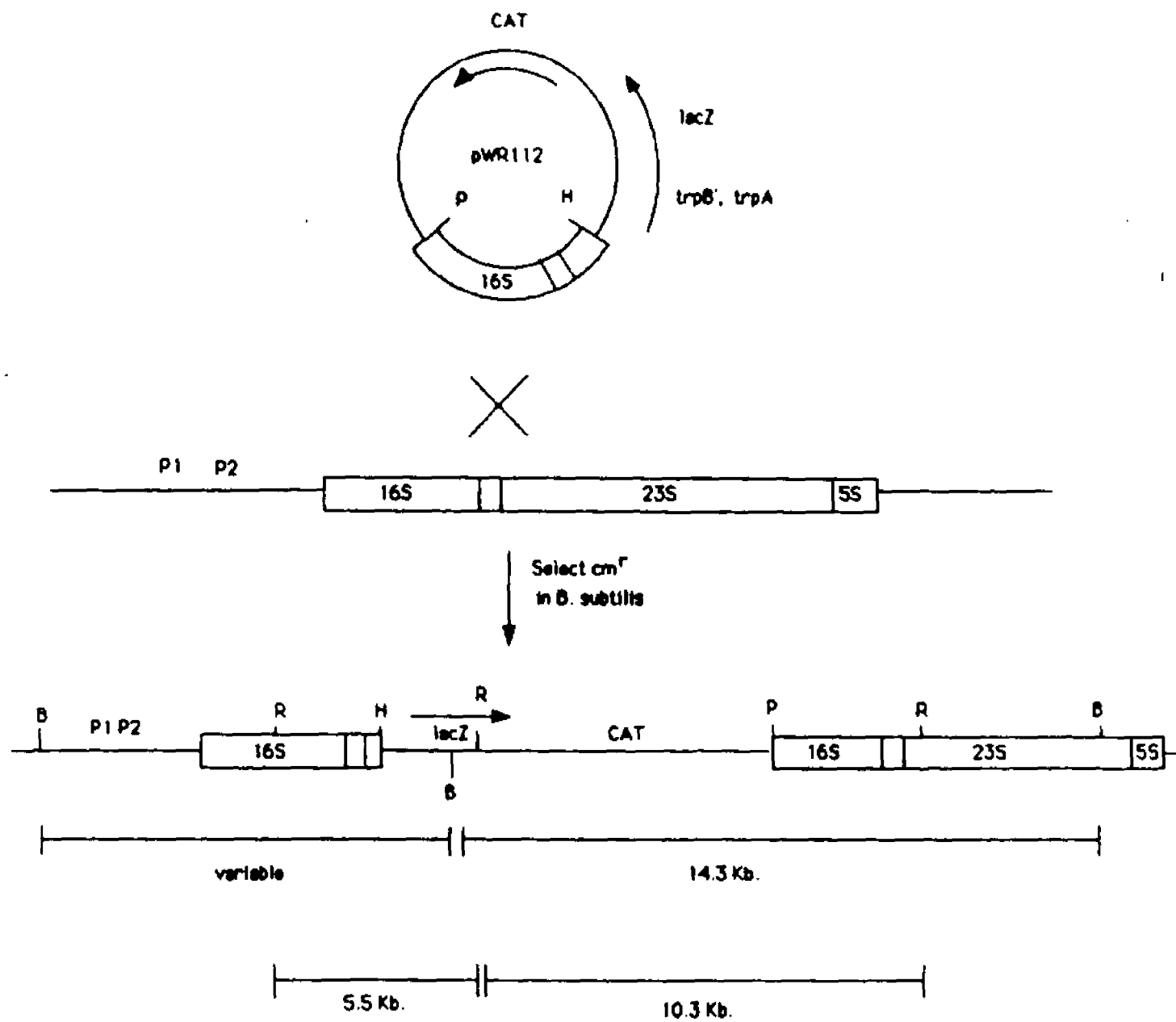


Figure 25. Southern Analysis of pWR112 Insertions.

Autoradiogram of Southern blot of pWR112 integrant DNAs restricted with BclI, electrophoresed through 0.75% agarose (5-7 days at 15mA), blotted and probed with PstI-EcoRI 23S rDNA sequences from pYR104. The DNA used is as follows:

1. SB25
2. SB25-42
3. SB25-41
4. SB25-96
5. SB25-77
6. SB25-43
7. SB25-100

A common 10.3Kb. fragment is found in lanes 2,3,5 and 7 (indicated by the arrow).

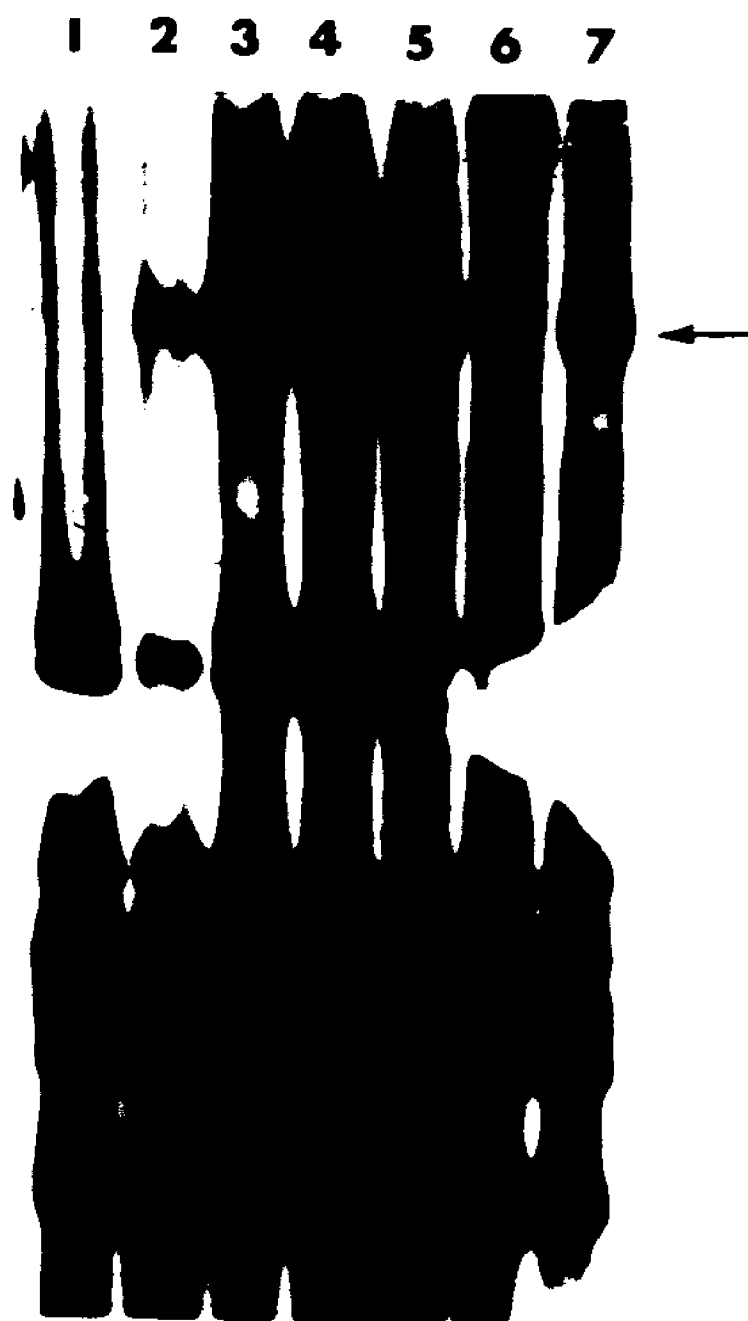


Figure 26. Southern Analysis of pWR112 Insertions Using a 16S Probe.

Autoradiogram of a Southern blot of pWR112 insertions. BclI restricted DNAs were run as described in Figure 25, blotted and probed with pWR103 containing 16S sequences. DNAs used are as follows:

1. SB25-41
2. SB25-42
3. SB25-43

The upper arrow indicates the position of the 14.3Kb. fragment released from pWR112 insertion strains which also hybridizes to 23S rDNA (Figure 25). The middle arrow indicates the position of the 8.2Kb. fragment released from strain SB25-42 (see text). Strain SB25-41 shows a missing 5.7Kb. homolog, but a more intense hybridization is evident just below the 5.7Kb. position (lower arrow).



Figure 27. EcoRI Analysis of pWR112 Insertions.

Autoradiogram of a Southern blot of EcoRI restricted integrant DNAs. DNAs were electrophoresed in 0.8% agarose overnight at 30mA, blotted and probed with pDEB-1 (Schreier and Sonenshein, 1986), which contains no rDNA sequences. DNAs are as follows:

1. SB25-191
2. SB25-41
3. SB25-96
4. SB25-77
5. SB25-43
6. SB25-100

The lower arrow indicates the 5.5Kb. fragment with vector homology (Figure 24) found in all strains representing the rrn-lacZ fusion. The upper arrow indicates the position of the 10.3Kb. fragment found in strains SB25-41,77,100, and 42 (not shown), found only in strains with no DNA duplications.

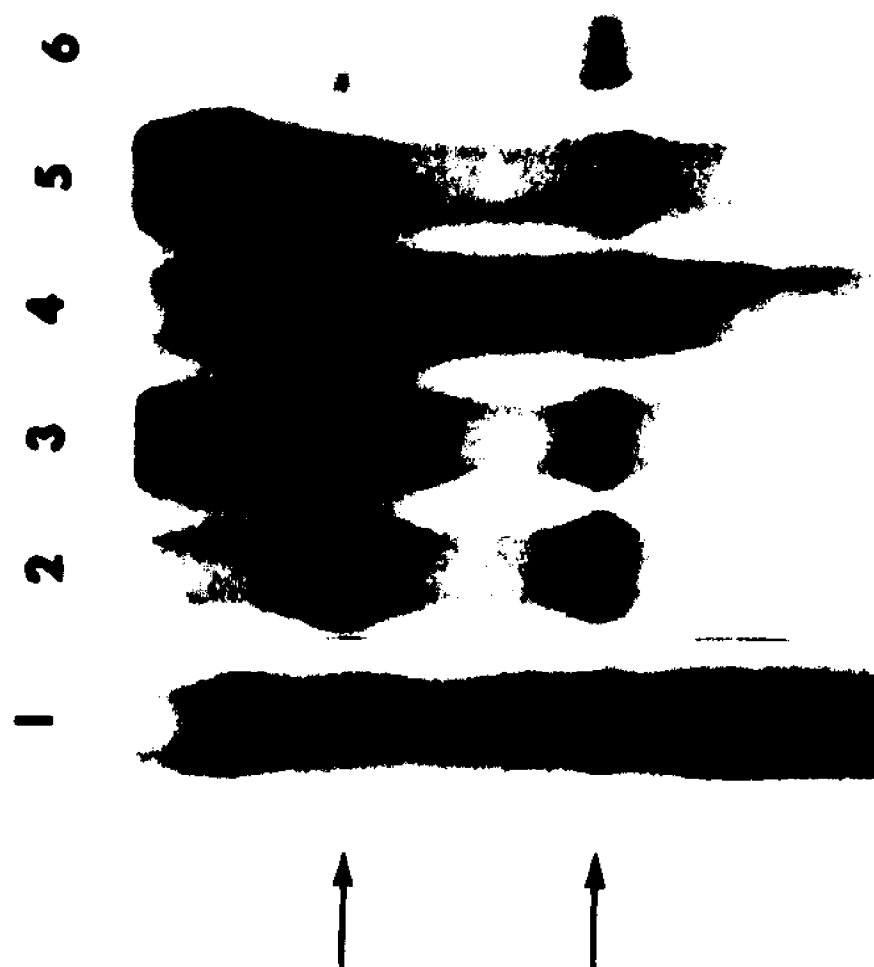
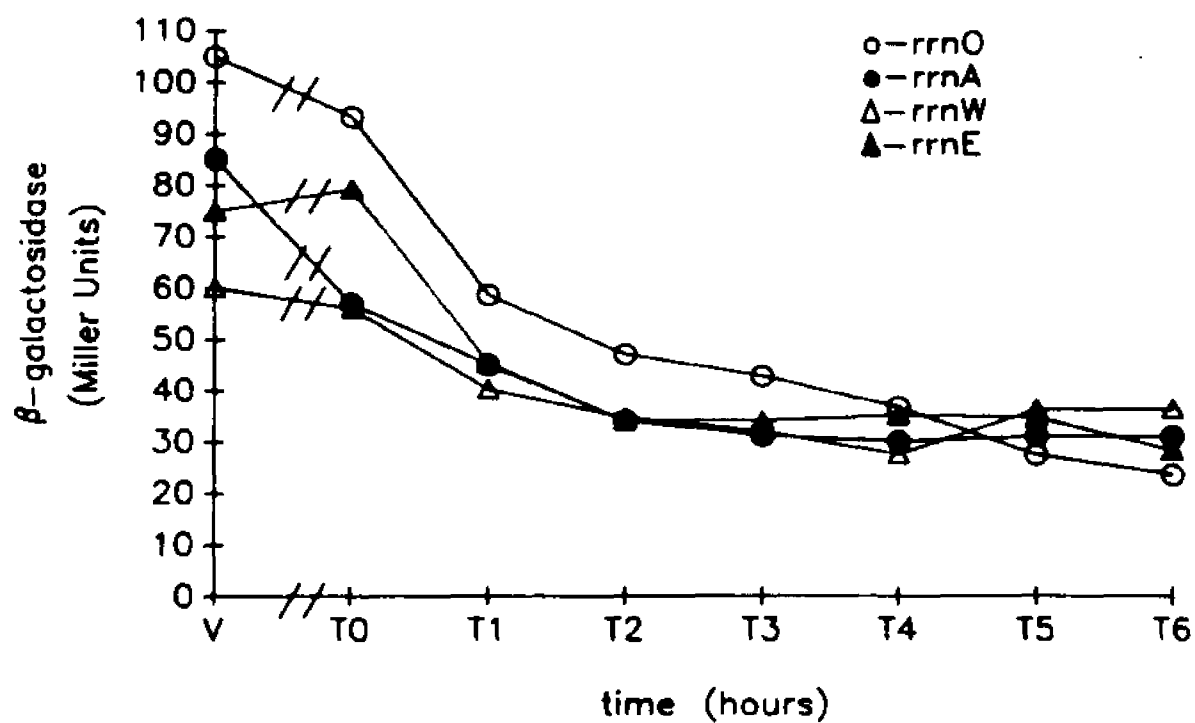


Figure 28. Activity of rRNA Operons During Sporulation.

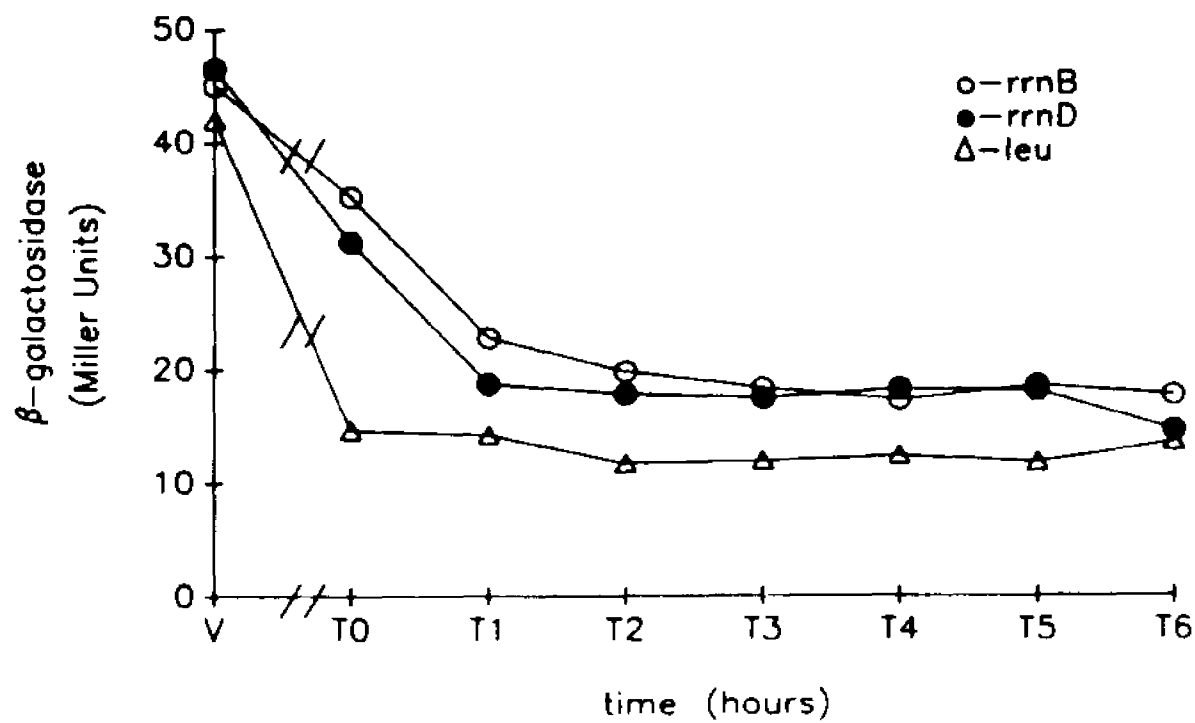
a) Plot of beta-galactosidase activity versus time for strains carrying rrn-lacZ fusions created by insertion of plasmid pWR112. The letter V indicates vegetative peak activity in SG medium; T_0 is the beginning of sporulation. The strains measured were: rrnO; SB25-42, SB25-191, rrnA; SB25-41, rrnW; SB25-96, rrnE, SB25-77.

b) Same plot as above for operons expressing lower amounts of beta-galactosidase. Strains measured were: rrnB; SB25-100, rrnD; SB25-43, SB25-80, leu; SB25-92.

a.



b.



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