CLONING AND EXPRESSION OF *BACILLUS SUBTILIS* RIBOSOMAL RNA GENE PROMOTERS IN *ESCHERICHIA COLI*

By

HENRICUS GERARDUS DENEER

B.Sc., The University of Manitoba, 1975
M.Sc., The University of Manitoba, 1981

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Department of Microbiology

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date Oct. 9, 1986
Abstract

In *E. coli*, ribosomal RNA (rRNA) is synthesized in proportion to the cellular growth rate squared, a phenomenon known as growth rate dependent regulation. The promoters of rRNA operons, which consist of two tandemly arranged -35, -10 regions about 120 bp apart, mediate this characteristic form of regulation. To provide insight into how this regulation is achieved and to extend previous observations from studies with *E. coli* to the rRNA operons of other species, the promoter region from the *B. subtilis* rrnB operon was cloned onto a transcription fusion plasmid such that the synthesis of chloramphenicol acetyltransferase (CAT) was directed by the *B. subtilis* promoters. Expression in *E. coli* showed that CAT specific activity increased in a growth rate dependent manner. The synthesis of CAT mRNA, the CAT mRNA half-life, and plasmid copy number were all measured directly to establish the validity of using CAT specific activity as a measure of promoter function. Only the downstream P2 promoter of the *B. subtilis* P1-P2 tandem pair was shown to be growth rate regulated; in contrast, the P1 promoter of the native *E. coli* rrnB operon was responsive to growth rate. Deletion of an A-T rich sequence upstream of the *B. subtilis* P1 promoter had no effect on the overall level of P1 or P2 transcription. These results indicated that the general mechanisms conferring growth rate regulation were conserved between *E. coli* and *B. subtilis*,
although differences were noted at the level of the individual P1 and P2 promoters.

In an attempt to develop a similar operon fusion system for use in *B. subtilis* a number of bi-functional shuttle vectors were constructed by fusing *B. subtilis* plasmids to an *E. coli* replicon carrying a promoterless catechol 2,3 dioxygenase gene. However, it was found that overexpression of this protein was lethal to *E. coli* cells and rRNA promoters could not be maintained unless their transcriptional activity was curtailed. Furthermore, such bi-functional vectors were highly unstable in *E. coli* or after transfer to *B. subtilis*. Nevertheless, data obtained using one of these vectors indicated that *B. subtilis* rRNA promoters did not possess the antitermination function associated with the analogous *E. coli* promoters, illustrating an additional functional difference between *E. coli* and *B. subtilis* rRNA promoters.
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<tr>
<td>Kb</td>
<td>Kilobase</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<td>dNTP</td>
<td>deoxynucleoside triphosphate</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
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<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGTA</td>
<td>ethyleneglycol-bis-(B-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
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<td>Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>chloramphenicol resistance</td>
</tr>
<tr>
<td>Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>ampicillin resistance</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenicol acetyltransferase</td>
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<td>l</td>
<td>liter</td>
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<td>ug</td>
<td>microgram</td>
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<tr>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>kanamycin resistant</td>
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<tr>
<td>Em&lt;sup&gt;r&lt;/sup&gt;</td>
<td>erythromycin resistant</td>
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<td>Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>tetracycline resistant</td>
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<td>A</td>
<td>absorbance</td>
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Literature Review

1. General Overview and Physiological Considerations

It has long been a tenet of bacterial physiology that if a cell suddenly finds itself in a nutritionally advantageous environment it can respond by increasing its growth rate. In order to do so it must first increase its capacity for protein synthesis. The immediate response is therefore one of an increased accumulation of ribosomes since these are key components in the protein synthesizing machinery of the cell. However, the ribosomes of *E. coli* are complex organelles composed of 53 different proteins and 3 different stable RNA molecules divided between 2 subunits (see Ref. 1 for review), whose synthesis must, from an efficiency standpoint, be closely regulated. Nevertheless, in recent years it has become apparent that the regulation of the RNA fraction of ribosomes is central not only to the question of how the synthesis of the numerous ribosomal components is coordinated, but also to how various nutritional and physiological conditions affect the accumulation of ribosomes as a whole.

The recognition of the importance of ribosomal RNA (rRNA) stems from the pioneering studies of Maaloe and co-workers (2) who first noted that a strict correlation existed between the rate of ribosome accumulation and the cellular growth rate.
(herein referred to as "u", the reciprocal of the cellular
doubling time expressed as doublings per hour). By studying the
macromolecular composition of bacterial cells as a function of
their growth rate they found that the relative proportion of RNA,
DNA, and protein changed as the growth rate increased; the ratio
of DNA to protein for example, remained essentially invariant,
but the ratios of RNA to DNA or RNA to protein increased dramatically.
In other words, the increase in the amount of RNA per cell (up to
97% of which is stable rRNA and tRNA as opposed to the more
labile messenger RNA) was proportionally much greater than the
increase seen for other macromolecules.

Later measurements showed that the actual number of ribosomes
per cell increased as a linear function of the growth rate (3),
implying that the increased demand for protein synthesis by
faster growing cells was met by an increase in the number of
ribosomes rather than an increase in their translational
activity. However, it was noted that the linear proportionality
between u and the number of ribosomes meant that the rate of
ribosome synthesis, obtained by dividing the proportion of the
cell mass which was ribosomes by the cell doubling time, must
increase as a function of $u^2$. In simple terms this could be seen
as being necessary if one imagined that faster growing cells must
pass on more ribosomes to each daughter cell in order to maintain
an increasing growth rate. But, as growth rate increased, the
cells had progressively less time in order to synthesize more
ribosomes unless the rate of this synthesis increased with the growth rate squared. Thus, while the fraction of cell mass occupied by ribosomes could vary from 15% at u=0.2 to 45% at u=2.5, the rate of ribosome synthesis could vary by more than 30-fold in this same range of growth rate (4). The proportionality between the cellular growth rate and the rate of ribosome (and ribosomal RNA) synthesis has been recognized as being a characteristic feature of the phenomenon known as "growth rate dependent regulation". This term, in a general sense, is analogous to the term "metabolic regulation" which had been coined to refer to controls that seemed tied primarily to the cellular growth rate rather than to the presence or absence of a particular nutrient (5). The problem of the growth rate dependent synthesis of ribosomal RNA and the mechanisms controlling this synthesis have been most thoroughly studied in E. coli although certain facets of this regulation have been clarified for other organisms such as B. subtilis as well.

2. Location, organization and structure of rRNA genes in E. coli.

As mentioned previously, the E. coli ribosome is comprised of three different RNA species, the 16S rRNA found in the 30S ribosomal subunit, and the 23S and 5S rRNA found in the 50S subunit. These three RNA species are transcribed poly-cistronically and in the order 16S-23S-5S, as was shown by the
isolation of mutants defective in the processing enzyme RNase III (6). Formation of the individual RNA species appeared to involve RNase - mediated processing events that occurred concomitantly with transcription (7). By using Southern hybridization to analyze restriction enzyme digests of total E. coli DNA, Kiss et al. (8) established that there were seven copies of rRNA operons per genome. The reason for this redundancy was not clear. Multiple copies may reflect the necessity for sufficient rRNA to meet the demands of very rapidly growing cells but whether exactly seven copies are needed is questionable. Ellwood and Nomura (9) have isolated a strain of E. coli in which one of the rRNA operons had been deleted but there was no obvious phenotypic change due to this mutation. Five of these seven rRNA operons have been mapped to an area between 70 and 90 min. on the E. coli chromosome (the remaining operons map near 5 and 57 min.), an area that also contains the genes for a number of other components of the transcription-translation apparatus (10). The clustering of rRNA operons near the origin of chromosomal replication may contribute somewhat to the increased rRNA synthesis at high growth rates because of an effective increase in gene dosage due to the increase in initiation of DNA replication at higher $u$. However, the overall regulation of rRNA synthesis cannot be explained in this way because it has been seen that this gene dosage increase only amounted to 20% as growth rate varied from 0.9 to 2.7 doublings.
per hour (9).

All seven rRNA operons were not identical. Heteroduplex analysis first indicated that the structure of different operons fell into two groups differing primarily in the sequence of the spacer regions between the 16S and 23S RNA coding regions (11). Each operon was found to contain either one or two tRNA genes within the 16S-23S spacer regions that were co-transcribed with the rRNA genes. Four of the operons carried the gene encoding Glu tRNA while the remaining three operons had genes for tRNA Ile and tRNA Ala (12, 13). In addition, three of the operons also carried co-transcribed tRNA genes distal to the 5S coding region (12).

All seven E. coli rRNA operons have been isolated on specialized transducing phage or plasmids, and six of the seven have been largely sequenced (for review, see 4 and 14). Sequence information, combined with data generated by analyzing the in vitro transcription products of cloned rRNA genes (15, 16), has revealed an unusual feature of the promoter region of all rRNA operons examined. Each operon contained a double, or tandem, promoter arrangement with the upstream or P1 promoter separated from the downstream or P2 promoter by about 120 base pairs. Each promoter of the tandem pair contained the basic elements identified for other E. coli promoters (see 17 for review), including a Pribnow box, a -35 region, and a transcriptional
start or "+1" site.

The availability of sequence data allowed some comparisons to be made in terms of the overall homology among six of the seven operons (4). All six operons contained a highly A-T rich region (22 out of 27 bp) between -35 and -62 (where +1 was the site of transcription initiation) of the upstream P1 promoter. The downstream P2 promoter also showed an A-T bias in this area (16 out of 27 bp) but not to the same extent as seen in P1. As well, the P1 promoter of all six operons had an identical 15 bp sequence (TCCCTATAATGC GCC) that included the Pribnow box. This conservation was not seen in the P2 promoter. The 120 bp region between P1 and P2 showed some areas of homology when any two operons were compared but overall there was no extensive conservation. The P1 promoter of all operons did however, contain a G-C rich "discriminator" region between +1 and +10. This region has been suggested to play a role in rRNA gene expression during the so-called stringent response (18), an adaptive response to conditions of amino acid starvation (see below). As well, all operons had a 67 bp region immediately downstream of the P2 transcriptional start site which may be required for the rRNA antitermination system (19, see below). Finally, an RNase III processing site (7) was identified in all rRNA operons in a region preceding the 16S RNA coding sequences. After this point, sequence homology became extensive with only 20
of the next 1541 bp being heterologous (1).

The far distal ends of the rRNA operons have also been examined in a number of cases and a number of probable transcription termination sites were recognized (20, 21, 22). All had a stem-loop structure followed by several U residues characteristic of rho-independent terminators (see Ref. 23 for review). Interestingly, the *E. coli* rrnB operon contained a double terminator arrangement (21) in which the first terminator was located adjacent to the 3' end of the 5S RNA gene and the second was about 175 nucleotides downstream. The significance of the double terminator is unclear since it has been shown that in *vivo* most if not all transcripts stop at the first terminator (24). In addition, such a tandem arrangement has not been noted in other rRNA operons (25).

3. Regulation of expression of rRNA genes.

It has become increasingly clear that ribosomal RNA operons are among the most complex operons yet identified, with the potential to be regulated at a variety of different points by a variety of different mechanisms. A key factor is the question of whether the overall regulation is at the level of transcription or at some post-transcriptional point. By comparing the rate of rRNA accumulation with the rate of *de novo* rRNA synthesis, Gausing (26) found that at moderate to fast growth rates the two
were virtually identical, indicating that there was little or no
degradation of excessively synthesized rRNA. Therefore, rRNA
accumulation was regulated primarily by modulating the rate of
synthesis. Furthermore, since the rate of rRNA chain elongation
was essentially constant at all growth rates (27), it appeared
that synthesis was regulated by modulating the frequency of
initiation of new chains (i.e. the frequency of initiation of new
rRNA transcripts). This conclusion was strengthened by the data
of Muto (28) who showed that the number of RNA polymerase
molecules per genome which were actively transcribing rRNA genes
increased in parallel with the rate of synthesis of rRNA as the
growth rate increased.

At slow rates of growth (below 0.3-0.4 doublings per hour),
a slightly different picture has emerged. Gausing (26) has shown
that the synthesis of rRNA exceeded the accumulation, with the
excess rRNA apparently being degraded. This became significant
at growth rates of 0.1 - 0.2 where up to 70% of the newly
synthesized rRNA was degraded. Thus, it appeared that rRNA
synthesis was regulated at the level of transcription initiation
in all but very slowly growing cells where degradation of newly
synthesized rRNA could play a significant role. The reason for
this dichotomy is presently not clear.

Since the promoters of rRNA operons appear to be the primary
control points in the growth rate dependent regulation of rRNA
synthesis, some of the factors which have been shown to influence the transcription of rRNA operons under different environmental conditions can be considered.

i) Upstream activation: A potentially important feature of stable RNA promoters is that in vivo they are transcriptionally much more active than the average E. coli promoter. Although rRNA genes comprise only 0.4% of the E. coli chromosome, up to 40% of newly synthesized RNA is rRNA (5). Several recent studies (29, 30, 31) have suggested that this enhanced level of expression is not due to the presence of two functional promoters rather than one, but is dependent on a stimulatory DNA sequence located upstream of the -35 region of P1. By performing a Tyr deletion analysis of the E. coli tRNA gene promoter, Lamond and Travers (32) first noted that removal of the wild-type sequence between position -40 and -98 caused a 10 to 12-fold decrease in promoter activity. However, these conclusions were reached by measuring the activity of a plasmid-borne Tyr galactokinase gene fused to the tRNA gene promoter and could be subject to artifacts due to the topological state of the plasmid versus the chromosome. Nevertheless, evidence that such an upstream stimulatory element was actually functional was obtained by showing that RNA polymerase could protect from DNase I digestion a region of the tRNA_Tyr promoter at least 75 bp upstream of the transcription initiation point (29). These
protection studies indicated that RNA polymerase could interact
with a region of DNA outside of the normal -35, -10 contact
points and suggested that binding of the polymerase molecule
to such secondary sites could serve to activate transcription
from the primary site via protein-protein interactions (29).

A second study which bears on the question of upstream
activation regions was performed by Bossi and Smith (30) using
His
the tRNA gene of Salmonella typhimurium. These authors showed
that a 3 bp deletion from -70 to -72 resulted in a 2-fold drop in His
transcriptional efficiency of the tRNA promoter. In addition,
the wild-type promoter element showed an altered electrophoretic
mobility in polyacrylamide gels. The wild-type promoter fragment
of 474 bp for example, migrated with an apparent size of about 550
to 570 bp. Deletion of the 3 bp fragment restored the normal
electrophoretic mobility. Other studies have shown that retarded
electrophoretic migration of DNA fragments could be correlated
with the presence of bends or kinks in the DNA helix (33, 34).
Bossi and Smith (30) have suggested that an aberrant DNA
conformation, upstream of the normal promoter region, was
somehow related to the transcriptional efficiency of the
promoter. This could result through a bending of the DNA around
the RNA polymerase such that the number of specific protein-DNA
contacts would be increased, thereby enhancing the initial
polymerase-promoter interactions.
Finally, Gourse et al. (31) have studied the E. coli rrnB promoter region by fusing it to a promoterless lacZ gene and monitoring B-galactosidase production after integration via a lambda vector of this construct into the bacterial chromosome. While chromosomal integration presumably eliminated any potential problems due to topological or copy number differences from the normal wild-type situation, it does leave open the question of the effects of chromosome location since the site of lambda integration is not in a region where rRNA operons are normally found (10). These authors have shown by deletion analysis that a region upstream of the rRNA P1 promoter between positions -51 and -88 could increase rRNA transcription at least 15-fold and also displayed a retarded electrophoretic mobility, similar to that seen by Bossi and Smith (30). Again, these results suggested the possibility that certain structural or conformational characteristics of the DNA region immediately preceding the classical procaryotic promoter element could function in a regulatory, or at least a stimulatory, capacity. Interestingly, all three of the upstream stimulatory sequences mentioned above fell into, or very near to, a highly A-T rich region commonly associated with growth rate regulated promoters. Whether the A-T rich region has any significance in terms of inducing the observed conformational changes or in some other regulatory capacity remains to be seen.
ii) Antitermination.

For most bacterial operons, the processes of transcription and translation have been shown to be coupled such that translating ribosomes prevent RNA polymerase from prematurely terminating transcription at intragenic cryptic terminators (see 35 for details). Such premature transcription termination has been shown to be dependent on an interaction between the termination factor "rho" and RNA polymerase (35). Ribosomal RNA operons are long, non-translated operons which would be expected to suffer from significant premature termination, but early experiments by Morgan (36) and Brewster and Morgan (37) showed that RNA polymerase molecules initiating at E. coli rRNA promoters could efficiently read through rho-factor dependent termination signals introduced by the insertion of transposons or non-coding DNA. This suggested that RNA polymerase initiating at these promoters might be modified so as to prevent premature termination without the need for transcription-translation coupling (38).

The bacteriophage lambda is known to use a transcription antitermination system as a regulatory mechanism during lytic growth (39). The lambda N-antitermination system has been shown to involve an elaborate interaction between a set of host proteins (Nus factors), RNA polymerase, and a site on the phage genome (nut locus) (40). Friedman and Gottesman (41) have proposed
three consensus sequences for the *nut* region, denoted Boxes B, A and C of which Box B is a region of dyad symmetry and is essential for antitermination (42) while Box A is a Nus protein recognition site (43). Recent sequence comparisons have shown that a Box B-A-C like sequence was located on a 67 bp fragment immediately downstream of the *E. coli* *rrnG* and *rrnB P2* promoters. Deletion experiments have shown that this sequence alone was sufficient to overcome rho-dependent transcription termination (44, 45) while Gourse *et al.* (31) have further pinpointed the critical regions as just consisting of Boxes A and C. Thus unlike the lambda system, Box B is not required for antitermination although it has been shown that at least the host Nus B protein is necessary (46). Whether these antitermination signals play any regulatory role in modulating rRNA gene expression is unclear; Gourse *et al.* (31) have suggested that their function is simply to ensure that RNA polymerase elongates through an untranslated operon. Nevertheless, the existence of such a system further underscores the overall complexity of rRNA operons.

**iii) Stringent control**

When bacterial cells encounter conditions of amino acid starvation they respond by halting further growth and rapidly adjusting their metabolism in such a way as to improve their chances for survival and allow quick recovery when the starvation
conditions are removed. These metabolic changes were collectively termed the stringent response by Stent and Brenner (47) and were seen to involve a wide range of cellular processes (reviewed in 48 and 49). For example, the replication of DNA was observed to be reduced, as was the biosynthesis of carbohydrates, lipids, nucleotides, and peptidoglycans. As well, the rate of intracellular proteolysis was increased, the transport of many precursor molecules across the cell membrane was inhibited, and the transcription of some operons such as his and lac was increased (49, 50, 51). The most prominent effect due to stringency was at the level of transcription of stable RNA genes, since the synthesis of the translational apparatus is a significant energy drain on rapidly growing cells (52). Overall, the transcription rate of these genes fell by 10-to-20 fold while the rate of bulk mRNA transcription dropped only 1-to-3 fold.

Most experimental data has shown that the selective regulation of rRNA can be modulated through effects on the initiation of transcription at stable RNA genes (53) although exactly how this occurs is unclear. The possibility that some effector molecule was involved was raised after it was noted that the intracellular concentration of a nucleotide, guanosine 5',3' tetraphosphate (ppGpp) increased from micromole to millimole amounts per cell concurrent with the decrease in stable RNA synthesis (54). Relaxed mutants of E. coli which failed to accumulate ppGpp continued to synthesize stable RNA during amino
acid starvation. A single genetic locus (relA) was thereby shown to encode a ribosome associated factor which synthesized ppGpp (55).

There is presently conflicting evidence as to how or even if ppGpp acts to modulate stable RNA activity during the stringent response. Nene and Glass (56) have isolated RNA polymerase mutants which produced a relaxed phenotype in vivo even though the mutant cells still made ppGpp after amino acid starvation. Similarly, Little et al. (57) reported the isolation of E. coli mutants in which the stringent response was induced at lower ppGpp concentrations; these mutations were found to alter the RNA polymerase enzyme. Both reports implicated a functional interaction between ppGpp and RNA polymerase and can be incorporated into a model for general stable RNA regulation proposed by Travers (58). This model will be discussed in detail in the following section.

Many investigators have reported that ppGpp specifically inhibited the synthesis of rRNA and tRNA in vivo (59, 60). Significantly however, a number of workers have failed to see the same effect (61). More recently, Kajitani and Ishihawa (62) used a mixed in vitro system whereby a number of different (i.e. stable and non-stable) gene promoters were placed in the same transcription reaction to show that low levels of ppGpp
significantly decreased rRNA transcription while not affecting the transcription from other promoters. Thus, most evidence favors the involvement of ppGpp in this aspect of stable RNA regulation, either directly at the DNA level or through the RNA polymerase. However, as stated previously, this is by no means clear as evidenced by the studies of Gallant et al. (63) who shifted *E. coli* from 23°C to 40°C and found that this induced a large accumulation of ppGpp, but surprisingly, the rate of rRNA synthesis increased as well. Based on these results, Gallant has suggested that ppGpp may not be the true effector of the stringent response but only increases co-incidentally during stringency and its observed *in vitro* effects may be due to its ability to mimic the actions of the true effector. It is apparent that much more work is needed to clarify this question.

As far as specific DNA sequences of stringently controlled genes are concerned, Travers and co-workers (64, 65) have suggested that at least two of three conserved features are necessary: i) an 8-10 bp G-C rich "discriminator" region located near the transcriptional start site; ii) a -35 region which deviated slightly from the TTGA of the consensus sequence; and iii) an upstream stimulatory sequence as discussed earlier. Using a system whereby the *E. coli* tRNA*_{Tyr}* promoter was joined to the *galK* gene, Lamond and Travers (65) demonstrated that a 4 bp mutation in the discriminator region abolished the stringent *Tyr* regulation of tRNA*_{Tyr} transcription *in vivo*, indicating that for
this gene at least, the G-C rich region was critical to stringency. In analogous studies, Sarmiento et al. (66) fused the P1-P2 promoter regions of the E. coli rRNA operon directly to a transcription terminator on a multicopy plasmid, eliminating most internal RNA structural elements, and directly assayed in vivo and in vitro transcripts on polyacrylamide gels. Interestingly, they found that only the upstream P1 promoter was subject to stringent regulation. Furthermore, both the P1 and P2 promoters contained a discriminator-like sequence near the transcriptional +1 site, indicating that a G-C rich sequence alone was not sufficient to confer stringent control. A more detailed mutational analysis of the P1 promoter element appears to be necessary to pinpoint the stringent control sequences.

4. Growth rate dependent regulation of rRNA operons.

1) DNA sequence requirements

All studies thus far have implicated the promoter region of stable RNA genes as being critical to a number of different forms of regulation. That growth rate dependent control was also mediated transcriptionally has already been implied by the data of Gausing (26). Direct evidence indicating that the promoter regions of rRNA and tRNA genes are necessary and sufficient for growth rate dependent regulation has only come with the advent of recombinant DNA technology. Most investigators have chosen to construct systems whereby the promoter region of interest is
fused to a marker gene whose product can be easily and quantitatively assayed, such as *lacZ* or *galK* (see 67 for review). The fusion is then introduced into the appropriate host organism either on a multicopy plasmid or on lysogenic phage and the transcriptional activity of the promoter monitored under various conditions by assaying for the expressed protein. The fusions discussed here and used throughout this thesis are properly termed "operon" or "transcriptional" fusions since the marker gene retains its native translational signals (ribosome binding site), as opposed to "gene" fusions which result in the synthesis of a hybrid protein (67).

Using this approach, Berman and Beckwith (68) first showed that the *lacZ* gene could be inserted into the *E. coli* tRNA gene such that the synthesis of B-galactosidase was subjected to the regulatory mechanisms which governed stable RNA synthesis. In this case, some of the fusions showed increased levels of B-galactosidase activity with increasing growth rate which paralleled the synthesis of stable RNA. Unfortunately, the methods used to generate *lacZ* fusions, based on phage Mu insertions, did not permit a precise localization of where within the tRNA gene the insertions had occurred. Interestingly, a number of their fusions did not exhibit a growth rate dependent increase in B-galactosidase activity. A more refined approach was taken by Ota et al. (69) who subcloned the promoter region
plus some 16S RNA coding sequences from the *E. coli* rrrN operon onto a lambda pgal8 transducing phage. Here again it was found that expression of the fused galactose operon increased as a function of cellular growth rate in a manner characteristic of rRNA synthesis, and was subject to stringent regulation as well. Since then a number of other studies have all reached the conclusion that only the promoter region of stable RNA genes was required to place a fused gene under growth rate dependent control (31, 70, 71, 72). Deuster et al. (71) have shown that a Leu DNA fragment of the tRNA promoter spanning residues -50 to +5, when fused to the galK gene on a multicopy plasmid, was sufficient to place galK expression under growth rate control.

The most detailed analysis of the critical sequence has been presented by Gourse et al. (31) who used chromosomally integrated lacZ fusions to the rrrE and rrrB promoter regions. By physically separating the upstream (P1) and downstream (P2) promoters they were able to show that only the P1-lacZ fusion gave the characteristic growth rate dependent increase in β-galactosidase activity; lacZ controlled by the P2 promoter showed no change in expression as growth rate increased. By progressively deleting both 5' and 3' flanking regions of the P1 promoter, they found that the DNA sequences between -51 and -4 relative to the P1 transcriptional initiation site were sufficient to confer a growth rate dependent response. The -35 region of P1 (between -51 and -20) appeared to be critical
because substitutions of the wild-type -10 region with a -10 sequence from a non-regulated (lac) promoter resulted in only a slight loss of regulatory capacity. Therefore, the role of the -10 region is somewhat undefined. Gourse et al. (31) have suggested that only the actual -35 region of P1 is critical for growth rate dependent regulation; however, their smallest regulated fragment (-51 to -20) still contained roughly 15 bp of the highly A-T rich 5' flanking sequences. Therefore, the possible importance of sequences outside of the -35 region cannot be excluded and more specific mutagenesis studies are needed to clarify this point. Nevertheless, as Travers (58) had previously pointed out, there does appear to be some conservation of sequences in the P1 promoter region of the seven E. coli rRNA operons, but this homology is largely confined to the -10 region. Therefore, the precise role of specific bases remains to be defined.

Support for the observation that the P1 and P2 rRNA promoters are individually and differentially regulated has also come from earlier studies by Cashel and co-workers (66, 73, 74). Rather than a traditional operon-fusion system as discussed above, these workers coupled the rrnA promoters to the rrnB transcription termination region (eliminating all internal sequences) in such a way that a small, easily visualized in vivo transcript was produced. Expression of the rrnA promoters could
be monitored by extracting total cellular RNA and quantifying the specific rRNA transcripts. In this way, Cashel's group (66, 73) observed that the overall activity or strength of the P1 promoter was about 3-fold greater than the P2 promoter and that only the P1 transcript appeared to increase as a function of increasing growth rate. Furthermore, Sarmientos et al. (74) have reported that neither P1 nor P2 transcripts were observed in stationary phase cells, although their method of detection (ethidium bromide staining of RNA in polyacrylamide gels) had limited sensitivity. Upon outgrowth from stationary phase the accumulation of P2 transcripts increased rapidly while P1 transcripts appeared only after a delay and took some time to reach their characteristic high levels. These results suggested that while P1 activity clearly predominated at moderate to fast growth rates and was regulated in response to growth rate, the normally weakly active P2 promoter might function as a means whereby rRNA synthesis could quickly resume in cells recovering from stationary phase (74).

It must be pointed out that most of the conclusions mentioned above were derived from the study of operon-fusion systems, some carried on multicopy plasmids, and therefore must be critically examined. Since any given rRNA operon is normally present as only a single copy per chromosome and gives rise to a non-translated product, there exists the possibility that measuring the translation product of a gene fused to an rRNA-derived
regulatory region could produce an artificial situation which could mimic a growth rate dependent response. Several potential artifacts include: a) differential translation of mRNA at high and low growth rate; b) differential stability of mRNA at different growth rates; or c) fluctuations in plasmid copy number as growth rate changed. These will be discussed further in the Results, Chapter 1. None of the studies discussed above established the validity of the fusion system by simultaneously determining the level of translated product and the level of fusion-specific mRNA as growth rate changed.

ii) Models for growth rate control of rRNA genes.

The major question now posed is how the concentration of ribosomes per cell is regulated in response to changes in environmental conditions (i.e. growth rate). As stated before, the concentration of ribosomes (and rRNA) increases approximately in proportion to the growth rate, \( u \), and thus the rate of synthesis increases with \( u \). It has been shown that the synthesis rate of rRNA is of considerable importance in the regulation of ribosome biosynthesis. In order to be effective, the mechanisms governing growth rate dependent regulation must firstly be able to sense the nutritional status of a cell's environment and secondly, be able to respond to this status by inducing the appropriate amount of rRNA transcription for that
growth rate and thereby produce the correct number of ribosomes. In general, three different models have been proposed for the regulation of stable RNA genes.

a) Maaloe (75) suggested a passive regulatory mechanism whereby the cell has only a limited capacity for transcription and that ribosomal and non-ribosomal promoters must compete for this capacity. This competition could switch in favor of ribosomal genes as growth rate increased because as the quality of the growth medium improved many of the pathways of intermediary metabolism and biosynthesis would become increasingly repressed. However, certain experimental observations are in conflict with this model. For example, Ikemura and Nomura (76) and Jinks-Robertson et al. (77) increased the gene dose of rRNA operons per genome by introducing plasmids containing rrnD or rrnB operons and showed that the level of expression from each individual chromosomal rRNA operon was subsequently reduced while there were no apparent changes in the transcriptional activities of other competing non-ribosomal genes. While the Maaloe model would predict that such a passive mechanism would influence the overall pattern of expression of every gene in the cell in a concurrent manner, this is clearly not seen in vivo.

b) Bremer and co-workers (78, 79) and Travers et al. (58, 80) have proposed similar models whereby stable RNA genes are
actively regulated via the action of positive or negative effector molecules. These effectors would presumably sense the nutritional conditions and then interact with DNA or RNA polymerase or both so as to induce the appropriate rate of transcription from rRNA operons. The favored effector molecule was ppGpp because of the inverse correlation between its concentration and rRNA synthesis during stringent control, as discussed in the previous section. Travers et al. (80) have presented evidence that the RNA polymerase holoenzyme could be fractionated by centrifugation through glycerol gradients into a number of different forms which had different preferences for various promoters. One form could initiate transcription efficiently at stable RNA promoters but relatively poorly at constitutive promoters; a second form had the opposite preference. Therefore, these workers have proposed that at least two different conformations of polymerase exist and that regulators of growth rate control such as ppGpp operate by changing the equilibrium between the form which can transcribe stable RNA genes and the form which cannot. At saturating levels of ppGpp (or some other effector), the polymerase would be in the form which prefers mRNA to stable RNA promoters. This model is supported by the finding that the E. coli alt1 mutation was phenotypically a mutant RNA polymerase having a higher affinity for the constitutive lac promoter and concurrently, a reduced affinity for the rrnX promoter in vitro (81); in other
words, an RNA polymerase with altered promoter preference. In addition, Muto (82) reported that crude protein factors, distinct from any RNA polymerase subunit, could preferentially stimulate rRNA synthesis several fold \textit{in vitro}. This was taken as evidence that certain factors could have a positive effect on rRNA transcription by shifting the balance between the two forms of polymerase. Whether positive effectors truly operate \textit{in vivo} is presently unclear. Nomura et al. (14) have pointed out that this direct effector model does not have an easy means for both assessing the nutritional status of a cell and also monitoring the results of its actions, in terms of responding to the final ribosome supply so as to fine-tune rRNA synthesis as conditions change.

c) For this reason, Nomura and co-workers (14, 77) have proposed an alternative model consisting of a feedback inhibition mechanism by which free, non-translating ribosomes inhibit the transcription of stable RNA genes. The ribosome itself may therefore be an autogenous regulator, sensing environmental conditions in a manner previously proposed by Lindahl and Zengel (4). This model was derived from studies by Jinks-Robertson et al. (77) who showed that an increase in the total number of rRNA operons per cell did not cause a corresponding increase in total rRNA synthesis. Instead, the overall rRNA synthesis rate was unchanged because the synthesis from each individual rRNA operon was decreased. In other words, a two to three-fold increase in
rRNA gene dosage resulted in a two to three-fold decrease in accumulation of tRNA and rRNA from individual operons such that the global rate of stable RNA synthesis was unchanged. More importantly, this effect was not seen if the extra rRNA operons which were introduced were deleted in the rRNA coding region such that full length rRNA transcripts could not be produced. In this latter case, the total rate of rRNA operon transcription (i.e. chromosomally encoded plus defective, plasmid encoded genes) increased in accordance with the increasing gene dosage. These results allowed Jinks-Robertson et al. to conclude that some product from intact rRNA operons was responsible for the maintenance of normal rRNA transcription rates. Either free rRNA, incomplete ribosomes, or complete ribosomes synthesized in excess of the amount appropriate for a given set of environmental conditions, would presumably feedback inhibit the transcription of stable rRNA operons.

Based on the observation that ribosome assembly-defective mutants of *E. coli* apparently overproduce rRNA and tRNA, Nomura et al. (14) have postulated that the negative regulatory factors are in fact surplus, non-translating ribosomes. This model has been tested in a number of studies by Nomura's group. Takebe et al. (83) developed a system in which the assembly of ribosomes was preferentially inhibited without inhibiting overall macromolecular synthesis. This led to a deficiency in free
ribosomes and a concurrent stimulation of rRNA and tRNA synthesis. Gourse et al. (84) constructed a conditional rRNA gene expression system by fusing the lambda P promoter/operator to the E. coli rrnB operon. The resultant overproduction of rRNA and free ribosomes was seen to produce a large repression of rRNA and tRNA synthesis from chromosomal genes. A definitive test however, has thus far been lacking - that is, it has not yet been possible to show a direct regulatory effect of ribosomes on transcription from rRNA promoters in vitro (77). It remains a distinct possibility that free ribosomes act indirectly by regulating some other factor which in turn is the true effector of rRNA operon control. In this regard, it is interesting to note that the study of Gourse et al. (84) showed that the overproduction of plasmid rRNA which resulted in a 2-fold inhibition of chromosomal rRNA transcription also correlated with a 30-50% increase in cellular ppGpp concentration. This would again implicate ppGpp in the control of stable RNA synthesis, but as pointed out by Gourse et al. (84) it could also be possible that the increase in ppGpp was the result rather than the cause of the observed inhibition of chromosomal rRNA transcription.

Finally, this model would predict a direct effect of free ribosomes (or some factor regulated by free ribosomes) on the rRNA promoter region so as to regulate the overall pattern of transcription. Gourse et al. (31) studying deleted rRNA
promoters in a lac fusion system as discussed previously, have found that the sequences critical for growth rate dependent control (between -51 and -20 of the P1 promoter) are also the sequences required in order that feedback inhibition effects are seen if the rRNA gene dose is increased. From these results, they have proposed that negative feedback regulation of rRNA promoters is responsible for the growth rate dependent synthesis of rRNA in E. coli. Whether this is truly the case remains to be experimentally determined.

In summary, two important points must be made. Firstly, the latter two models discussed above are not entirely incompatible but require an intermediary factor to relate the cellular ppGpp concentration to the free ribosome pool. The important but largely overlooked work of Gallant et al. (63) involving the effects of temperature upshift on ppGpp concentration and rRNA synthesis suggests that such an intermediary factor may indeed exist. Secondly, it is clear that there are several features unique to stable RNA genes, features that are found at the DNA sequence level that allow them to be recognized as targets for the growth rate regulatory mechanisms. Therefore the study of rRNA promoter regions and their regulation in other organisms could be important in terms of identifying and characterizing these unique features.
5. Ribosomal RNA operons in *Bacillus subtilis*

While there is a wealth of information concerning the organization, structure and regulation of stable RNA genes in *E. coli*, comparatively little is known about the situation in other species. The Gram-positive, endospore-forming organism *Bacillus subtilis* has been the subject of most studies in this area for a number of reasons. Because it is a sporulating organism, it follows what is in effect a primitive differentiation pathway directed by a cascade of modified RNA polymerase molecules recognizing different, specific sets of promoters (85). An analysis of the transcriptional regulation of stable RNA genes as well as other genes could therefore point to new mechanisms related to development and cellular differentiation. Secondly, *B. subtilis* could represent a potentially useful system for the expression of heterologous genes since an efficient protein secretion system operates in this organism. Finally, in contrast to other Gram-positive organisms, *B. subtilis* has been relatively well characterized genetically (86), which can facilitate the more detailed study of specific genes and operons.

6. Location and structure of *B. subtilis* rRNA genes.

As in *E. coli*, the ribosomal RNA genes of *B. subtilis* are organized as transcriptional units in the order 16S, 23S, 5S RNA (87). While no large precursor RNA has been observed, precursors
to the individual rRNA's could be detected after pulse labelling, indicating that some post-transcriptional processing events were required (88). Early density transfer experiments carried out by Oishi and Sueoka (89) and Smith et al. (90) showed that about 80% of the DNA that specifically hybridized with ribosomal RNA was replicated very early, indicating that this DNA was clustered near the origin of chromosome replication. The remaining 20% replicated considerably later, suggesting the presence of a second chromosomal locus. This tight clustering was confirmed by Chow and Davidson (91) who used electron microscopy to study the heterduplexes formed after denatured B. subtilis DNA was allowed to renature. Their data suggested that there were 7 to 10 copies of ribosomal genes on the chromosome and that each was homologous but separated from the others by heterologous spacer DNA sequences. More recently, by using Southern blotting to examine the distribution of chromosomal DNA sequences homologous to radiolabelled ribosomal RNA, a number of authors have found that B. subtilis probably possesses as many as 10 rRNA operons (92, 93, 94, 95). By cloning rDNA sequences onto plasmids and allowing these to integrate into the homologous chromosomal regions, La Fauci et al. (96) were able to map the location of seven of these operons, bringing the number of mapped operons to nine (86). A cluster of six operons \( \text{rrn} 0, A, G, H, I, E \) was mapped to a region between 0 and 15, \( \text{rrnD} \) lay between 60 and 75, and the remainder in a second cluster between 200 and 275.
Thus, the rRNA operons of *B. subtilis* apparently were clustered to a greater extent than seen for *E. coli* operons.

By analyzing cloned rDNA sequences and chromosomal patterns of homology, Stewart et al. (93) were able to construct detailed physical maps for some of the operons. They found that the linkage between distinct operons could be very tight - some were apparently separated by as little as 100 bp, and others by less than 500 bp. In addition, they were able to divide the rRNA operons into two groups which differed only in the size of the intergenic 16S-23S spacer regions. Whereas all *E. coli* rRNA operons carried tRNA genes in this intergenic spacer, only two of the ten *B. subtilis* operons were found to contain intergenic tRNA genes and both of these consisted of tRNA and tRNA sequences. Interestingly, other workers have reported the clustering of tRNA genes between tandem rRNA gene sets in *B. subtilis* or as large groups located immediately distal to the 5S RNA determinants (92, 94). Green and Vold (97) for instance, have recently reported the sequence of a cluster of 21 tRNA genes located distal to the *B. subtilis* *rrnB* operon; in contrast, the largest known cluster in *E. coli* contains only 7 tRNA genes (98).

The significance of such tight clustering of stable RNA genes in *Bacillus* is unclear, although Vold (99) has speculated that this could be an evolutionary feature common to Gram-positive organisms. It is clear, however, that the stable RNA
genes of \textit{B. subtilis} differ fundamentally in organization from their \textit{E. coli} counterparts. As outlined above, this is reflected in the extremely tight clustering of rRNA and tRNA genes, the relative location of tRNA determinants in relation to rRNA gene sets, and in the gene dose of rRNA operons.

7. Promoter regions of \textit{B. subtilis} rRNA operons.

To date, the complete DNA sequence of only one \textit{B. subtilis} rRNA operon has been determined (102). In addition, the sequence of the promoter region of a second rRNA operon has been determined (103). As with \textit{E. coli}, the 5' flanking region of the \textit{B. subtilis rrnB} operon showed the presence of distinct tandem promoter elements separated by about 100 bp. Interestingly, the second operon, on a plasmid called 14B1, appeared to possess only a single promoter element which showed homology to the downstream P2 promoter of the other operons (103). It was also noted that the spacing between the -35 and -10 region of the 14B1 promoter was a less than optimal 18 bp, compared to 17 bp for the other rRNA promoters. These differences call into question the overall efficiency of this promoter and its role \textit{in vivo}, especially in view of the fact that its chromosomal location was as the second set of a tandem operon arrangement.
Finally, an inspection of the DNA sequences surrounding the tandem promoters of the *Bacillus* rRNA operon revealed the presence of a stem-loop structure downstream of the P2 promoter transcription initiation site and thought to be part of a post-transcriptional processing site (103). Also, a G-C rich sequence showing homology to the discriminator sequence thought to be involved in the stringent response in *E. coli* was seen in the area of the +1 site of the *B. subtilis* rRNA P1 promoter, although not for the P2 promoter. Furthermore, the sequence upstream of the P1 promoter in all *B. subtilis* rRNA operons was seen to be extremely A-T rich (37 out of 44 bp), as noted previously for *E. coli* rRNA operons (4).

8. Regulation of stable RNA synthesis in *B. subtilis*.

Comparatively little is known about the synthesis of stable RNA in general, and the regulation of rRNA operons in particular, in *B. subtilis*. A direct analogy to *E. coli* may be an oversimplification because of the ability of *B. subtilis* to follow an alternate developmental pathway culminating in the formation of endospores. As has been shown by numerous authors, the process of sporulation involves the differential expression of a subset of genes whose transcription is dependent on the presence of different sigma factors associated with the core RNA polymerase (85). Sigma factors in general have been shown to be required for the specific recognition of promoter sites on DNA by
RNA polymerase (105), and as such are key components in the initiation of transcription. The promoters for stable RNA genes have been shown to have sequences potentially recognized by the major sigma-43 containing RNA polymerase of vegetative cells (106). Since this form of polymerase is known to be present at all stages of sporulation and vegetative growth, it has been speculated that stable RNA genes are not in fact subject to differential regulation of transcription caused by variations in available sigma factors (99). This has not been experimentally tested however.

Despite the availability of cloned rRNA and tRNA genes, few transcriptional studies have been done. Stewart and Bott (103) used S1 mapping techniques to demonstrate that the rrnB promoters functioned in vivo and provided evidence that the upstream P1 promoter was the less active promoter of the pair, although the relative hybridization efficiencies were potentially complicating factors here. Similarly, Vold and Green (107) used S1 mapping to analyze the transcription products from a cluster of 21 tRNA genes after introduction of these genes into E. coli. Despite the fact that this was a heterologous system, it was shown that mature tRNA's having the primary sequence of E. subtilis tRNA's could be transcribed and processed into functional isoaccepting species, thereby emphasizing the similarities between these two organisms in this regard.
However, studies attempting to relate the transcriptional activity of *B. subtilis* stable RNA gene promoters to variations in cellular growth rate have not been done.

As far as the overall regulation of the *Bacillus* transcriptional-translational machinery is concerned, a number of studies have suggested a pattern similar to that seen in *E. coli*. For example, the RNA polymerase of *Bacillus* was found to be similar in subunit structure to *E. coli* RNA polymerase although there were slight differences in the relative sizes of the B and B' subunits and, as mentioned above, in the number of different sigma subunits associated with the polymerase (108). By quantitating the amount of B subunit per cell, Leduc et al. (109) calculated that the number of RNA polymerase molecules per *B. subtilis* cell increased with increasing growth rate and to roughly the same extent as seen in *E. coli*. Furthermore, these authors noted that *Bacillus* apparently had an excess of RNA polymerase at all growth rates, as did *E. coli*, although the excess at lower growth rates was significantly greater than that seen in *E. coli*. It was suggested that this could be a reflection of certain differences in transcriptional control mechanisms, possibly related to the presence of multiple sigma factors in *Bacillus*. Finally, it was shown in this study that the total RNA content of *Bacillus* cells increased in a logarithmic manner as growth rate increased. That the growth rate dependent increase in total RNA was due predominantly to
ribosomal RNA was later shown by Webb and Spiegelman (110) who measured the hybridization of radiolabelled RNA to a cloned rRNA gene probe. Again, as with \textit{E. coli}, the synthesis of rRNA in \textit{Bacillus} followed a classical growth rate dependent response, supporting the idea that overall growth rate regulation was similar in these two organisms. Data obtained through measuring the rate of rRNA synthesis in phage SP01-infected cells suggested that \textit{B. subtilis} possessed a stable, ribosomal RNA-specific RNA polymerase distinct from the polymerase used for transcription of non-stable RNA genes (110). How such a polymerase could be regulated or how this was related to the overall mechanism for growth rate dependent control of stable RNA genes in \textit{Bacillus} was unclear.

Finally, as with \textit{E. coli}, \textit{B. subtilis} has been shown to produce the modified nucleotide ppGpp under conditions of amino acid starvation (162) and the stimulus required for the \textit{in vivo} synthesis of this nucleotide may be the same as that in \textit{E. coli} (see Ref. 163 for review). Despite the fact that relaxed mutants (those that fail to shut down stable RNA synthesis during amino acid starvation) have been isolated in \textit{B. subtilis} (163), there is as yet no clear cut indication that the mechanistic details of the stringent response is similar in \textit{B. subtilis} and \textit{E. coli}. It must be noted however, that the GC rich sequences located at the origin of transcription of stringently controlled genes and thought to be involved in mediating the stringent response (64),
are homologous to sequences found around the transcription initiation site of the *B. subtilis* rrnB P1 (but not the P2) promoter (see sequence in Ref. 103). Whether this has any bearing on the stringent regulation of this operon remains to be determined.

9. Rationale for present studies.

From the preceding discussion, it is clear that in a broad sense the regulation of rRNA synthesis in both *E. coli* and *B. subtilis* is similar although there is evidence that subtle differences may exist. With the availability of isolated and characterized *Bacillus* rRNA operons it is now possible and necessary to begin a detailed analysis of the regulation of such operons *in vivo*. Such an analysis would hopefully point to further similarities or differences in how stable RNA synthesis is controlled in two widely divergent organisms and possibly provide an independent test of some of the models currently proposed to explain the general mechanisms of growth rate dependent regulation. We have chosen to follow an approach that has yielded much information concerning the expression of *E. coli* rRNA genes; namely the fusion of *Bacillus* rRNA promoter regions to genes whose expression can subsequently be easily and quantitatively monitored. As will be discussed below, such an approach is a valid means of assessing the transcriptional activity of isolated promoters under a variety of environmental
conditions provided the appropriate control experiments are carried out. Our primary objectives therefore, were to isolate the promoter region from a *B. subtilis* rRNA operon, fuse it to an assayable structural gene and monitor its expression in *E. coli* and *B. subtilis* as a function of growth rate. The expression, if possible, of *Bacillus* rRNA genes in *E. coli* may provide an indication of the evolutionary divergence of this component of the transcription-translation system among procaryotes as well as provide insights into the mechanistic details of growth rate dependent control. Similarly, the expression of these genes in a homologous *Bacillus* background is necessary to ascertain whether subtle regulatory differences exist between this organism and *E. coli*. 
Materials and Methods

1. Bacterial strains and plasmids

Strains of bacteria used throughout this thesis and plasmids other than those constructed here are listed in Table 1. Plasmids constructed during the course of this work will be described in the Results.

2. Purification of DNA

i) *E. coli* plasmid DNA - large scale

Plasmid DNA was isolated from *E. coli* HB101 by cesium-chloride density gradient centrifugation of cleared lysates prepared by the method of Clewell and Helinski (111) as described by Dobinson and Spiegelman (112). Centrifugation of cleared lysates was carried out in a Beckman 70.1 Ti rotor for 36-40 hr. at 108,000 x g. The supercoiled plasmid DNA band was removed with a syringe and 18G needle, extracted with H<sub>2</sub>O-saturated n-butanol and dialyzed overnight against 4 l of 10 mM TRIS-Cl (pH 7.5), 1 mM EDTA, 10 mM NaCl. The concentration of plasmid DNA was determined by measuring the A<sub>260</sub> (1 A<sub>260</sub> = 50 ug/ml of DNA).

ii) *E. coli* plasmid DNA - small scale.

For the rapid analysis of recombinant clones, an alkaline lysis "miniprep" procedure was used which routinely yielded 2-3
## Table 1  Bacterial Strains, Plasmids, Phage

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E.coli</strong> HB101</td>
<td>F^- pro leu thi lacY endA rpsL20 recA araI4 galK2 xyI5 mtlI supE44 hsdR hsdM</td>
<td>T.Beatty</td>
</tr>
<tr>
<td><strong>E.coli</strong> C110</td>
<td>F^- str31 tsx33 sup37 supE44 proA2 his4 argE3 galK2 araI4 xyI5 mtlI thrI leuC thiI lacY</td>
<td>R.E.W.Hancock</td>
</tr>
<tr>
<td><strong>E.coli</strong> JM101</td>
<td>supF thiI Δ(lac proA,B)/F' traD36 proA,B lacI92 M15</td>
<td>R.Miller</td>
</tr>
<tr>
<td><strong>B.subtilis</strong> 168 Marburg</td>
<td>trpC2 thr5</td>
<td>C.Price</td>
</tr>
</tbody>
</table>

## Plasmids and Phage

<table>
<thead>
<tr>
<th>Host</th>
<th>Properties</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;B.subtilis phage</td>
<td>encodes toluene degradative enzymes</td>
<td>P.putida, G.Hegeman, 145</td>
</tr>
<tr>
<td>B.subtilis</td>
<td>&lt;B.subtilis &gt;</td>
<td>H.Whiteley, 161</td>
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<tr>
<td>M13 cloning vector</td>
<td>&lt;E.coli &gt;</td>
<td>R.Miller, 122</td>
</tr>
<tr>
<td>lambda ilv5</td>
<td>lambda phage carrying E.coli rRNA operon</td>
<td>E.coli, P.Dennis, 137</td>
</tr>
</tbody>
</table>
ug of plasmid DNA pure enough for most purposes. The procedure of Maniatis et al. (113) was followed except for minor modifications. The cells from 2.0 ml of a saturated overnight culture were lysed, phenol extracted, and the plasmid DNA was ethanol precipitated as described (113). The precipitated pellet was dried under vacuum, then resuspended in 100 ul of sterile H 0. 5 ul of DNase-free pancreatic RNase was then added and the solution incubated at 37 C for 15 min. Next, 31 ul of 7.5 M ammonium acetate and 300 ul of isopropanol were added and the tubes placed on ice for 15 min. to allow the plasmid DNA to precipitate. The tubes were then spun for 10 min. at full speed in an Eppendorf centrifuge, the pellet was resuspended in the required volume of sterile H 0 (not exceeding 50 ul) and used as needed.

iii) B. subtilis plasmid DNA

Plasmids from B. subtilis were purified by cesium-chloride density gradient centrifugation after the cells were lysed using a sodium dodecyl sulfate/NaCl procedure as described by Gryczan (114). Overnight cultures of B. subtilis were used to inoculate 1 litre volumes of Luria broth plus the appropriate antibiotics. Cultures were grown at 37 C with aeration for 6 hr., after which the cells were harvested, lysed, and the plasmid DNA prepared for density gradient ultracentrifugation (114). Centrifugation was at 108,000 x g
for 36 hr. in a Beckman 70 Ti rotor. Bands corresponding to plasmid DNA were removed and treated as described for \textit{E. coli} plasmid DNA purification.

\textbf{iv) \textit{B. subtilis} chromosomal DNA.}

\textit{B. subtilis} 168 was grown in 1 l of Luria broth for 8 hr. at 37 C. Cells were harvested, converted to protoplasts, lysed, and the chromosomal DNA spooled out as described by Rodriguez et al. (115). Chromosomal DNA was resuspended in TE buffer (10 mM TRIS-Cl, pH 8.0; 1 mM EDTA) to a final concentration of 1 mg/ml.

\textbf{v) \textit{Pseudomonas putida} TOL plasmid.}

\textit{Pseudomonas putida} mt-2 carrying pWWO (TOL) was grown with aeration at 30 C for 12 hr. in 1 l of Luria broth. Cells were harvested and 3 g aliquots were lysed using the Sarkosyl-deoxycholic acid/polyethylene glycol method of Johnston and Gunsalus (116). DNA was fractionated by equilibrium banding in cesium chloride-ethidium bromide density gradients as described (116). Supercoiled plasmid DNA was removed with a syringe and 18G needle, extracted, and dialyzed as described above.

3. Transformation of \textit{E. coli} and selection of recombinants.

\textit{E. coli} HB101 was made competent for transformation by
the CaCl₂ method as described by Maniatis et al. (113) but with the following modifications. A single colony was used to inoculate 1.0 ml of Luria broth and grown overnight as a stationary culture. This was then diluted to 100 ml with fresh Luria broth and grown to an A₅₅₀ of 0.5. Twenty-five ml of this culture were harvested by centrifugation at 4000 x g for 5 min. in a Sorval SS-34 rotor. The cell pellet was resuspended in 12 ml of cold CaCl₂-TRIS-Cl solution (113) and incubated on ice for 15 min. The cells were again pelleted as above and gently resuspended in 1.5 ml of cold CaCl₂-TRIS-Cl solution. Finally, 2 ml of cells were pipetted into sterile 1.8 ml Eppendorf tubes and left on ice for 6-18 hrs. Transformation of these competent cells was carried out by addition of 0.1-0.5 ug of purified plasmid DNA or a ligation mixture, incubation of the cells on ice for 30 min., followed by a 42°C heat shock for 2.5 min. Finally, 1.0 ml of Luria broth was added to the transformed cells and they were allowed to grow for 1 hr. at 37°C before plating.

Transformed cells (0.1 ml) were plated on Luria agar plates supplemented with the appropriate antibiotics (50 ug/ml ampicillin for pBR322 derivatives) and allowed to grow overnight at 30°C. When selection was based on the xylE phenotype, transformed cells were plated, allowed to grow into colonies (1-2 mm.) then sprayed with a 0.5 M aqueous solution of catechol using
an aerosol sprayer. $\text{XylE}^+$ colonies turned a bright yellow color within 15 sec. of spraying. Single recombinant clones were picked, grown in 2.0 ml of antibiotic-supplemented Luria broth and analyzed for plasmid content by the alkaline lysis method described above.

4. Transformation of $\text{B. subtilis}$.

$\text{B. subtilis}$ 168 was made competent and transformed using a modification of the method of Dubnau (117, C. Price, personal communication). A loopful of $\text{B. subtilis}$ cells was spread onto a fresh TBAB agar plate (1% Bacto-Tryptone, 0.3% Beef Extract, 0.5% NaCl, 0.5% glucose, 1.5% agar, pH 7.2) and grown overnight at 30°C. Three or four colonies from this plate were used to inoculate 10 ml of pre-warmed C-1 media (0.6% KH$_2$PO$_4$, 1.4% K$_2$HPO$_4$, 0.1% Na$_2$C$_6$H$_7$O$_7$, 0.2% (NH$_4$)$_2$SO$_4$, 0.5% glucose, 0.13% MgSO$_4$, 0.02% Casamino acids, 50 μg/ml tryptophan) in a 125 ml side-arm flask. This gave a starting optical density of about 5 Klett units (Klett-Summerson spectrophotometer, #54 green filter). The flask was shaken vigorously in a 37°C water bath and growth followed by taking Klett readings every 30 min. The time at which the culture started to deviate from logarithmic growth was noted and the flask was allowed to continue shaking for exactly 1 hr. beyond this point. At this time, 0.5 ml of cells was pipetted into a sterile 1.8 ml Eppendorf tube, pelleted by centrifugation at full speed for 2 min., and then
resuspended in 1.0 ml of prewarmed C-2 media (as C-1, except that Casamino acids were 0.01% and tryptophan was 5 ug/ml). One-tenth of a ml of cells were transferred to 13 x 100 mm. test tubes, placed on a tube roller at 37 C, and shaken for 40 min. at which time the cells were maximally competent. For subsequent transformation by plasmid DNA, the efficiency of transformation was enhanced by adding 1 ul of 100 mM EGTA to the competent cells. After this, 0.5 - 1.0 ug of plasmid DNA was added and the cells shaken at 37 C for another 30 min. To allow expression of plasmid genes, 400 ul of Luria broth was added to the transformed cells and incubated for 1 hr. The transformation mixture was then plated on fresh TBAB agar plates containing the required antibiotics at 5 ug/ml final concentration and incubated at 37 C overnight.

5. Cloning procedures.

The recombinant DNA techniques used here were essentially as described by Maniatis et al. (113). Minor variations will be noted here.

1) Restriction endonuclease digestion of DNA.

Restriction endonucleases were obtained from New England Biolabs or P.L. Biochemicals and were used with the appropriate
buffers as recommended by the supplier. For density gradient purified DNA, generally 5 units of restriction enzyme were used per ug of DNA and incubated for 1.5 hr at the appropriate temperature. For DNA prepared by the alkaline lysis miniprep method, it was found that more enzyme and longer incubation times were necessary to give complete digestion. Therefore, in these cases, 10-15 units of restriction enzyme were used per ug of DNA and incubation was extended to 2.5-3.0 hr.

ii) Blunting of 5' and 3' extended ends.

DNA restriction fragments (1-2 ug) which had 5' extended ends were made flush by the addition of 2 ul of each dNTP (2 mM stock solution) in a final volume of 30 ul. One-tenth volume of 10X Klenow buffer (113) was added, followed by 2 units of the Klenow fragment of E. coli DNA polymerase (New England Biolabs). The reaction was incubated for 20 min. at room temperature, after which the Klenow enzyme was inactivated by heating to 65 C for 15 min.

Fragments (1-2 ug) with 3' extended ends were made flush by addition of T4 DNA polymerase and the appropriate dNTP exactly as described by Maniatis et al. (113).

iii) Dephosphorylation of DNA.

In ligation reactions where it was desired that vector self-ligation be minimized, the terminal 5' phosphates of the
vector molecules were removed with calf intestinal alkaline phosphatase. Generally, 22 units of phosphatase were used per ug of vector DNA. Reaction conditions and inactivation of phosphatase were as described by Maniatis et al. (113).

iv) Ligation conditions

Ligations were carried out on a total (vector plus insert) of 0.5-0.7 ug of DNA where the molar ratio of insert to vector was approximately 1:1. The DNA was resuspended in 13 ul of sterile H 0, to which 1.5 ul of 10x ligation buffer were added (1x was 50 mM TRIS-Cl, pH 7.8; 10 mM MgCl ; 15 mM dithiothreitol; 1 mM spermidine; 0.5 mM ATP; 50 ug/ml bovine serum albumin). To this, four units of T4 DNA ligase (New England Biolabs) were added and the reactions incubated at 12 C overnight or 16-18 C for 6 hrs.

v) Exonuclease Bal-31 reaction conditions.

All reactions were carried out at 30 C in a total volume of 50 ul. Nuclease Bal-31 (New England Biolabs) was used at 1 unit per ug of linearized plasmid DNA under the buffer conditions specified by the manufacturer. Incubation time varied between 30 sec. and 3 min. after which the reactions were stopped by the addition of cold EGTA to a final concentration of 0.02 M. Samples were then diluted 3 times with sterile H 0 and precipitated with 1/10 volume of 3 M sodium acetate (pH 5.0) and
2 volumes of 95% ethanol. Specific applications of Bal-31 exonuclease and further characterization of DNA will be discussed in the Results.

6. Analytical and preparative gel electrophoresis.

1) Agarose gels.

Supercoiled or restriction endonuclease treated plasmid DNA was analyzed on agarose "minigels" consisting of either 0.7% or 1.0% agarose (Bio-Rad) in TBE buffer (0.089 M TRIS Base; 0.089 M boric acid; 0.002 M EDTA), with ethidium bromide added to 0.001%. Gels were cast on 5.0x7.5 cm. glass slides, loaded with DNA mixed in sample running dye (2x TBE, 50% glycerol, 0.25% xylene cyanole FF, 0.25% bromophenol blue), and electrophoresed in TBE buffer at 60 mA until the bromophenol blue dye reached the bottom of the gel DNA was visualized by placing the gel on a UV transilluminator (Ultra-Violet Products, Inc.). When desired, gels were photographed with transmitted UV light using a Kodak MP-4 camera equipped with a Vivitar VMC orange filter and Polaroid Type 667 film.

ii) Polyacrylamide gels

Plasmid DNA restriction patterns were analyzed on native polyacrylamide gels (180 x 140 x 1.5 mm.) of either 4% or 8% acrylamide, prepared using a stock solution of acylamide (30% acrylamide: 0.8% N,N,-methylene bisacrylamide) in TBE buffer,
0.05% (w/v) ammonium persulfate, and polymerized with 0.1% (w/v) N,N,N,N ‑tetramethylethyline diamine. DNA in bromophenol blue sample running dye (as above) was electrophoresed at 130 volts, then stained in 0.1% ethidium bromide for 15 min. before viewing with a UV transilluminator.

DNA sequencing gels were cast between 200 x 360 x 0.35 mm. glass plates as described above except that the stock acrylamide solution was 43.5% acrylamide: 1.5% N,N ‑methylene bisacrylamide, urea was added to 7 M final concentration, and the TBE buffer contained 0.05 M TRIS base, 0.05 M boric acid, and 0.001 M EDTA. Samples were mixed with loading buffer (40% formamide, 25 mM EDTA, 0.05% xylene cyanol FF, 0.05% bromophenol blue), denatured by boiling for 2 min., and electrophoresed at 1200 volts.

Protein samples were electrophoresed on sodium dodecyl sulfate (SDS) ‑ polyacrylamide gels using the conditions and buffer system of Laemmli (118). Gels were either 18% acrylamide or exponential gradients from 12% to 20% acrylamide with a 3% stacking gel. Samples were mixed with SDS loading buffer (0.005 M TRIS ‑Cl, pH 6.8; 2% SDS; 0.2% B ‑mercaptoethanol; 20% glycerol; 0.25% bromophenol blue), boiled for 3 min., then electrophoresed at 100‑120 volts. Gels were stained overnight with Coomassie Brilliant Blue R, then de‑stained with a solution of 25% glacial acetic acid and 25% ethanol for 6 hr.
7. Isolation of DNA fragments from gels.

i) agarose minigels

For DNA fragments greater than about 500-600 bp, a trough elution method was used whereby a small trough was cut out of the gel directly in front of the leading edge of the DNA band. The trough was filled with TBE buffer and the DNA electrophoresed into it while following its progress with a hand-held UV light source. The recovered DNA was precipitated by the addition of 1/10 volumes of 3 M sodium acetate (pH 5.0) and 2 volumes of 95% ethanol. After incubation at -70 C for 30 min, the DNA was pelleted by centrifugation. The DNA pellet was dried under vacuum and resuspended in a small volume of H O.

ii) polyacrylamide gels.

DNA fragments smaller than about 500 bp were isolated from polyacrylamide gels by excising the appropriate band with a scalpel and chopping the gel slice into fine pieces. The gel fragments were covered with 0.5 ml of elution buffer (0.5 M ammonium acetate; 1 mM EDTA pH 8.0) and placed in a 65 C water bath overnight. The buffer was then removed and passed through a small plug of cotton to remove small gel fragments. The DNA was subsequently recovered from the supernatant buffer by ethanol precipitation as described above.
8. Nucleic acid sequencing.

The endpoints of Bal-31 generated deletions were sequenced by the chemical method of Maxam and Gilbert (119). Either an EcoR1 or BamHI end was radiolabelled with \[ {^{32}}P \]dATP or \[ {^{32}}P \]dGTP respectively, using the Klenow subunit of \textit{E. coli} DNA polymerase I. Reaction conditions were as described earlier except that 10 uCi of the appropriate labelled nucleotide were used per microgram of DNA. Chemical modification and cleavage reactions were performed under the conditions described by Maxam and Gilbert. Products of the reactions were electrophoresed through 12% or 20% polyacrylamide-urea gels as described above.


Gels with radiolabelled DNA were placed on glass plates wrapped with Saran Wrap, then overlaid with a sheet of 3M Hi Lite X-ray film. Gels were kept in the dark at 4 C for the appropriate time of exposure then developed according to the manufacturer's instructions. To reduce exposure time for some gels, an intensifying screen (Dupont Cronex Lightning-Plus) was placed on top of the X-ray film and exposed at -70 C.

DNA sequencing gels were dried onto Whatman 3 MM filter paper using a Bio-Rad Slab Gel Drier prior to autoradiography.
Protein gels were first subjected to fluorography by soaking the gel in an autoradiography enhancer (En Hance-Dupont) according to the manufacturer's instructions. Enhanced gels were then dried onto filter paper as described above before autoradiography.

10. Southern blotting and hybridization of DNA.

DNA samples were run on agarose minigels as described above, photographed, and prepared for transfer to nitrocellulose paper (Schleicher and Schuell, BA85) essentially as outlined by Maniatis et al. (113). The only deviation from the published procedure was that the initial acid depurination was eliminated. Transfer was allowed to proceed for 6-8 hr., after which the nitrocellulose filter was baked in a 65°C oven overnight.

Hybridization probes were prepared by nick translation of isolated DNA fragments or plasmids as described by Maniatis et al. (113). Generally, 5 units of _E. coli_ DNA polymerase (New England Biolabs), 10 uCi of [³²P]dCTP and 10 uM of each remaining dNTP were used per ug of DNA. The reaction was incubated at 14°C for 90 min., then terminated by heating to 65°C. Approximately 1.6 x 10⁹ CPM of probe DNA were used per hybridization reaction.

Hybridization of radiolabelled probe DNA to
nitrocellulose-bound DNA was as described by Southern (120). Prehybridization (6 hr.) and hybridization (18 hr.) reactions were performed in heat-sealable bags at 65 C in a buffer consisting of 200 ug/ml Ficoll, 200 ug/ml polyvinyl pyrrolidone, 200 ug/ml bovine serum albumin, 10 ug/ml thymidine, 0.25% SDS, 3 x SSC (1x was 0.15 M NaCl, 0.0015 M Na citrate, pH 7.0). After hybridization, the nitrocellulose filters were washed four times in 2 x SSC plus 0.5% SDS at 65 C and four times in 2 x SSC, dried briefly, then overlain with X-ray film and autoradiographed as described above.


Single stranded hybridization probes were constructed by cloning the structural gene for chloramphenicol acetyltransferase (CAT) into M13mp18. A 773 bp TagI restriction fragment containing the entire CAT structural gene was isolated from pBR328 (121) and blunt-ended. M13mp18 replicative form DNA, made as described by Messing (122) was digested with SmaI and ligated to the TagI fragment under the reaction conditions given above. The ligation mixture was used to transfect E. coli JM101 which had been CaCl₂-treated as described above. Both orientations of the 773 bp CAT gene were obtained in this way and the clone corresponding to the "reverse" orientation (i.e. non-complementary to the mRNA) was used as the blank in subsequent hybridization reactions.
Large quantities of single-stranded M13-CAT DNA were prepared by scaling up the "miniprep" procedure described by Messing (122). A 4 l culture of infected JM101 yielded approximately 2 mg of single-stranded DNA as determined spectrophotometrically (1 A = 40 ug/ml single-stranded DNA).

12. RNA - DNA hybridizations.

i) isolation of $[^3H]$-labelled RNA.

The procedure of Daniels and Bertrand (123) was followed. Plasmid-containing derivatives of E. coli HB101 were grown at different rates until a Klett value of 20-25 was reached. Ten ml aliquots of cells were labelled for 1 min. by addition of 10 uCi of $[^5,6-H]$ uridine per ml (45 Ci/mmol, Amersham). Incorporation was stopped by rapidly pouring the entire 10 ml culture into a Corex tube containing an equal volume of crushed, frozen stop solution (10% w/v sucrose; 20 mM TRIS-Cl, pH 7.3; 5 mM MgCl; 20 mM NaN; 400 ug chloramphenicol/ml). Labelled cells were harvested by centrifugation, resuspended in 3 ml of stop solution without sucrose, and 200 ug lysozyme/ml and 10 ug DNase I/ml were added. The cells were then subjected to 4 cycles of freezing and thawing and lysed by the addition of 35 ul of 3 M sodium acetate (pH 5.0) and 0.15 ml of 10% (w/v) SDS. After heating to 65 C for 5 min., the samples were extracted with 4 ml of buffer-saturated phenol. RNA was precipitated with 2 volumes
of cold 95% ethanol, resuspended in 1 ml of 0.3 M sodium acetate (pH 5.0), precipitated again with ethanol and finally resuspended in 0.4 ml of buffer (10 mM TRIS-Cl, pH 7.4; 300 mM KCl; 1 mM EDTA). The amount of RNA was quantitated spectrophotometrically (1 A$^3_{260}$ = 40 ug/ml RNA) and the incorporation of [H] - uridine was determined by precipitating 5-10 ug of RNA with cold 5% (w/v) trichloroacetic acid, collecting the precipitate on glass-fibre filters and counting by liquid scintillation.

ii) preparation of single-stranded (SS) DNA filters.

Approximately 1.5 mg of SS M13 CAT DNA was diluted to 10 ml in 0.1 x SSC and slowly filtered through a 115 mm. nitrocellulose filter (Schleicher and Schuell, BA85). After washing with 50 ml of 0.1 X SSC, the filter was air dried, baked at 65 C overnight and then smaller (6 mm.) filters were punched out using a cork borer. At the concentration of DNA initially filtered, each 6 mm. filter was calculated to contain 4 ug of SS M13-CAT DNA.

Filters containing lambda ilv5 DNA were prepared in exactly the same way except that the diluted lambda DNA was denatured by addition of NaOH (0.33 M final concentration) prior to filtration. In both cases, the extent of DNA retention to filters was assessed by monitoring the A$^260$ of the initial solution and comparing this to the flow-through filtrate.
iii) [H] RNA-DNA hybridization.

Hybridizations were carried out in 1.8 ml Eppendorf tubes (0.5 ml reaction volume) at 65°C for 18 hr. Generally, each reaction contained 3 M13-CAT filters, 1 "blank" M13-CAT filter (non-complementary orientation), and 2x10^4 CPM of trichloroacetic acid-precipitable radioactivity in 2xSSC plus 0.2% SDS. Filters were washed, RNase treated and counted in a toluene-based scintillation fluid as described by Daniels and Bertrand (123). Levels of CAT mRNA were calculated by subtracting the radioactivity bound to blank filters from that bound to M13-CAT filters and expressing the result as a percentage of input trichloroacetic acid-precipitable radioactivity.

13. Determination of mRNA half-life.

i) Functional half-life.

*E. coli* HB101 carrying the appropriate plasmid was grown under different nutritional conditions so as to achieve growth rates of about 0.5 hr^-1 (see section 15 (i) below). Ten ml of the appropriate media was inoculated from an overnight culture, shaken in a 37°C water bath, and growth monitored with a Klett-Summerson spectrophotometer. At a Klett value of approximately 40 (Green filter), 0.3 ml of the culture was added to a pre-warmed 1.8 ml Eppendorf tube containing 5 uCi of [35S]methionine and incubated for 3 min. at 37°C. At the same time, rifampicin
(200 ug/ml final concentration) was added to the remainder of the culture. 0.3 ml aliquots were withdrawn at 20 sec. intervals for 100 sec., added to 5 uCi of \(^{35}\)S]methionine as before and incubated for 3 min. At the end of this labelling period, an excess of unlabelled methionine (60 ug/ml final concentration) was added to each tube and incubation was continued for 1 min. After this, the tubes were rapidly chilled on ice, centrifuged for 3 min., and the cell pellet resuspended in 30 ul of SDS loading buffer (see above). Samples were boiled for 2 min., sonicated for 30 sec. using the microtip of a Fisher model 300 sonicator, boiled for another 3 min. and applied to an SDS-polyacrylamide gel in 20 ul aliquots. Electrophoresis, staining, de-staining, and autoradiography were as described earlier.

ii) Chemical half-life.

Chemical half-life was determined directly by monitoring the decay of hybridizable CAT mRNA following inhibition of transcription initiation by rifampicin. The method used was essentially as described by Daniels and Bertrand (123). Cultures of plasmid-containing E. coli HB101 were grown at different growth rates and when a Klett value of 20-25 was achieved, the cultures were labelled for 1 min. with 10 uCi of \(^{3}\)H]uridine per 3 ml. Further incorporation of \(^{3}\)H]uridine into RNA was stopped by addition of 200 ug of rifampicin per ml and 0.8 ug of uridine per ml. Ten ml samples of the culture were removed immediately prior
to, and 30, 60, and 90 sec. after, rifampicin addition and rapidly pipetted into crushed, frozen stop solution as described above. [H]RNA was extracted as described earlier. The levels of CAT mRNA were determined by hybridization to filter-bound M13-CAT SS DNA as described above.


Plasmid copy numbers in cells growing at different rates were determined by the dot-blot hybridization method of Adams and Hatfield (124). Portions of the cell extract used for chloramphenicol acetyltransferase assays (see below) were filtered onto nitrocellulose filter paper (Schleicher and Schuell, BA 85) using a Bio-Rad dot blot apparatus. A volume equivalent to 10 μg of total protein was filtered for each individual cell extract. As a standard, different known amounts of sonicated, denatured pKK232-8 DNA were also placed on the same piece of nitrocellulose filter. As a probe, pKK232-8 DNA was first linearized with SmaI, then nick translated as described before. Hybridization and washing were performed as described for Southern blots, using a large excess (2-3 μg/filter) of nick translated pKK232-8 DNA. Filters were exposed to X-ray film, then the individual dots on the paper were punched out and counted for Cerenkov radiation. The amount of plasmid DNA per 10 μg of cell extract protein was then calculated after comparison to a standard curve obtained from the known standard
DNA. Absolute number of plasmid copies per cell was not calculated, but instead, results were expressed as nanograms of plasmid DNA per microgram of total protein in the sonicated cell extract.

15. Growth rate studies and enzyme assays.

Plasmid-carrying strains of *E. coli* HB101 were grown under various nutritional conditions so as to achieve a growth rate of $0.5 - 1.5 \text{ hr}^{-1}$. Incubation temperature always remained constant at 37°C. At the appropriate culture density, cells were harvested and cell-free extracts prepared in order that plasmid-encoded enzymatic activity could be assayed.

i) Media and growth conditions.

For all experiments, cells were grown in AB minimal salts media (125) supplemented with a vitamin-amino acid mixture (thiamine, 20 ug/ml final concentration; proline, 50 ug/ml; leucine, 50 ug/ml; serine, 50 ug/ml; threonine, 60 ug/ml; tryptophan, 60 ug/ml; arginine, 50 ug/ml; histidine, 50 ug/ml) and either chloramphenicol at 50 ug/ml or ampicillin at 50 ug/ml depending on the plasmid carried by the cells. To achieve differences in growth rate, this media was also supplemented with: 0.4% (final concentration) glucose, 1% Bacto-tryptone, 0.2% $B^{-1}$ Bacto-yeast extract for growth rate ($u$) from 1.2-1.6 hr. ; 0.5%
glycerol, 0.1% Bacto-yeast extract for $u = 0.9 - 1.2$ hr. ; 0.5% sodium succinate, 0.4% Bacto-tryptone for $u = 0.7 - 1.0$ hr ; 0.5% sodium acetate, 0.1% Bacto-yeast extract for $u = 0.4 - 0.7$ hr.

To ensure that all cultures were in logarithmic growth at the time aliquots were taken for enzyme assays, the following protocol was adopted. The appropriate media was dispensed in 20 ml amounts into 250 ml side-arm flasks and kept at 4°C until use. Meanwhile, a Luria broth culture of the strain to be tested was serially diluted to at least $1 \times 10^{-7}$ in the same media in which subsequent growth was desired. These dilutions were then incubated at 37°C for 14-18 hr. After this time, some of the less diluted cultures showed dense growth but those cultures of higher dilution which presumably had started from only a few cells showed only faint growth. These cells, which were still in logarithmic growth were used to inoculate the pre-warmed side-arm flask containing the same media and grown with vigorous aeration at 37°C. Growth was monitored until a Klett reading of 20-30 (Green filter) was reached, at which point 15 ml of the culture was placed into a 25 ml Corex tube and rapidly chilled on ice. The cells were then pelleted by centrifugation at 4000 x g in a Sorval SS-34 rotor and assayed for enzymatic activity.

ii) Catechol 2,3-dioxygenase assay.

The enzyme was assayed essentially as described by
Zukowski et al. (127). The cell pellets were washed once with AP buffer (0.04 M NaHPO$_4$, 0.16 M Na$_2$HPO$_4$, 10% v/v acetone), then resuspended in 4 ml of AP buffer and sonicated for 1.5 min. ("65" setting) using the intermediate size tip of a Bronwill Biosonic sonicator. A cell-free extract was prepared by centrifugation at 12,000 x g for 15 min. in a Sorval SS-34 rotor. Catechol, 2,3-dioxygenase was assayed by adding 25-100 ul of cell-free supernatant to a tube containing: 2.5 ml H$_2$O, 0.3 ml AP buffer, 100 ul of a 0.1 M aqueous catechol solution. Upon addition of the supernatant, the reaction mix was transferred to a 1 cm. path length cuvette and placed in a temperature controlled (37°C) dual-beam spectrophotometer. The increase in absorbance at 375 nm due to conversion of catechol to hydroxymuconic semialdehyde was followed. Enzyme activity was normalized to the protein content of the supernatant, as measured by a modified Lowry assay (127). One unit of activity was defined as a change of 1 x $10^{-3}$ A units per minute per milligram of total protein.

iii) chloramphenicol acetyltransferase assay.

The method of Shaw (128) was used. Cell pellets were washed once with EB buffer (50 mM TRIS-Cl, pH 7.8, 30 mM dithiothreitol), resuspended in 5 ml of EB buffer and centrifuged as described above. A reaction mix consisting of 100 mM TRIS-Cl, pH 7.8; 0.1 mM acetyl-CoA; 0.4 mg/ml 5,5'-dithiobis-2-nitrobenzoic acid; and 0.1 mM chloramphenicol was made up and
used as a blank in a dual-beam spectrophotometer. One ml of the reaction mix was placed in a second cuvette, allowed to equilibrate to 37 C, and 10-50 ul of cell-free supernatant was added and the cuvette inverted several times to mix the contents. The increase in A was followed and chloramphenicol acetyl-
transferase activity was normalized to the protein content of the supernatant as described above. One unit of activity was defined as a change of $1 \times 10^{-3}$ A units per minute per 410 milligram of total protein.
Chapter 1

Cloning and expression of *B. subtilis* rrnB promoters in *E. coli*

Introduction

This chapter describes the isolation and cloning of the promoter region from the *B. subtilis* rrnB operon as well as the *E. coli* rrnB operon. The initial stages of this work were designed to determine whether in fact *B. subtilis* rRNA promoters were expressible in *E. coli* and if so, whether this expression was growth rate dependent in a manner as had previously been shown for *E. coli* rRNA promoters (31, 72, 73).

A number of vegetative genes from *B. subtilis* (those recognized by the sigma-43 RNA polymerase) have been expressed in *E. coli* and Void and Green (107) have shown that the promoter for a *B. subtilis* tRNA gene cluster is active in *vivo* in *E. coli*. While the *B. subtilis* rrnB promoter region is structurally similar to promoters recognized by *E. coli* RNA polymerase (103), it has not as yet been directly shown that these promoters are transcriptionally active in a heterologous *E. coli* host. It must be noted however, that the difficulties encountered by some workers in cloning *Bacillus* rRNA fragments on multicopy plasmids was presumptive evidence that these promoters are active in *vivo* (103).
Results

Cloning of *B. subtilis* rRNA promoters.

The plasmid pKM-1 was constructed as an expression vector for the cloning of unusually strong promoters (129). It consists of a promoterless galactokinase (*galK*) gene and an upstream polylinker region into which promoter-bearing fragments can be inserted. The presence of promoters inserted into the polylinker can be detected by activation of the *galK* gene. Finally, the lambda-phage tr1 transcription terminator was placed between the polylinker and the *galK* gene. This is a weak, rho-dependent terminator which allows only about 5-10% of the upstream transcripts to read through (129). Previous attempts at cloning rRNA or other strong promoters on multicopy plasmids in *E. coli* were unsuccessful because of severe plasmid instability (130). Presumably, this was due to interference with plasmid replication because transcripts emanating from the cloned promoters could read-through into the ori region. Terminator-containing vectors are therefore necessary as a means of preventing read-through transcription from high-activity promoters and thereby enhancing plasmid stability.

Using pKM-1, Stewart and Bott (103) cloned two contiguous *EcoR1* restriction fragments of approximately 1900 and 1100 bp
from the 5' end of the *B. subtilis* rrnB operon, to create pGS227. By sequencing, the larger fragment was found to contain the rrnB tandem promoters as well as about 1200 bp upstream of them. The plasmid pGS227 was digested with EcoRI, the 1900 bp promoter fragment isolated and subcloned into the EcoRI site of pKM-1 to give pHD1.8 (see Fig. 1A). Most of this 1900 bp fragment had been sequenced by Stewart and Bott (103) and it was found to contain a single BstE11 restriction site located 123 bp beyond the start of the 16S RNA coding sequence, about 1400 bp from the 5' end of the fragment (see Fig. 3A). This asymmetrically located BstE11 site and a PstI site located 754 bp upstream of the EcoRI cloning site of pKM-1 (129) were used to orient the insert relative to the galK gene on pKM-1. Simultaneous digestion with BstE11 and PstI would be expected to produce fragments of 2154 bp and 4046 bp if the insert was oriented such that the tandem promoters initiated transcription into the galK gene, and fragments of 1320 bp and 4880 bp if the reverse orientation was correct. As seen in Fig. 1B, two fragments of about 2000 bp and 4000 bp were produced, indicating that the former orientation was correct.

Although transcription proceeded into the galK gene if the rRNA promoters were active in vivo, no indication of galK expression was observed in *E. coli* strain C110 (galK) transformed with pHD1.8. This was assessed by growth of transformants on either McConkey-galactose or EMB-galactose
Figure 1. Structure and orientation of pHDI.8.

A. Structure of pHDI.8. A 1900 bp EcoRI fragment from pGS227 (103) was cloned into the EcoRI site of pKM-1. The start of the 16S RNA coding region is indicated. The lambda tRl transcription terminator is shown proximal to the galK gene. Abbreviations: P, PstI; E, EcoRI; B, BseEII; H, HindIII.

B. Orientation of the 1900 bp insert. Lane A - pHDI.8 undigested, Lane B - pHDI.8 digested with EcoRI, Lane C - pGS227 digested with EcoRI. Digested DNA was electrophoresed through a 1% agarose gel. The position of the 1900 bp EcoRI rrnB fragment is indicated.

Lane D - pHDI.8 digested with PstI and BseEII, Lane E - Ï¿29 digested with HindIII as molecular weight markers. Digested DNA was electrophoresed through a 1.2% agarose gel. Size of the Ï¿29 fragments, in base pairs, is indicated on the right. The presence of two fragments of 2154 and 4046 bp in Lane D was used to derive the orientation of the 1900 bp insert (see text).
indicator media. Therefore it was reasoned that either the promoters were not functional in this system, or some downstream element, either in the 16S RNA portion of the cloned insert or in the lambda terminator placed before the galK structural gene, was preventing expression of the galactokinase protein. In order to facilitate further manipulations of the Bacillus rrnB promoter elements, the pHD1.8 insert was used as a source of smaller DNA fragments carrying only the rRNA promoters, and a different expression vector was chosen. This expression vector was the plasmid pKK232-8 (131) which differed from pKM-1 in that it carried a promoterless chloramphenicol acetyltransferase (CAT) gene and it did not require an internal transcription terminator to maintain plasmid stability. Instead, a set of highly efficient terminators derived from the E. coli rrnB operon had been placed at the 3' end of the CAT gene so as to prevent read-through transcription into the plasmid ori region (Fig. 2). Furthermore, transcription terminators had been placed upstream of the CAT gene so as to prevent nascent transcripts emanating from the pBR322 portion of the vector from reading through into the CAT gene. A multiple cloning site polylinker placed between the upstream terminator and the CAT gene provided a means of inserting DNA fragments. The CAT gene encoded the enzyme chloramphenicol acetyltransferase which inactivated the antibiotic chloramphenicol by an acetylation reaction (128). Since this gene lacked a functional promoter but retained the
Figure 2. Structure of pKK232-8.

Stippled areas denote sequences derived from pBR322. 5S, T1, T2 refer to the 5S RNA gene and the downstream T1 and T2 transcription terminators derived from the E. coli rrnB operon. The promoterless chloramphenicol acetyltransferase (CAT) gene is shown as "Cam". Multiple cloning sites proximal to the CAT gene are used for insertion of promoter fragments to give a chloramphenicol resistance phenotype. Note the presence of an additional transcription terminator (T1) proximal to the multiple cloning sites.

Abbreviations: Ps, PstI; E, EcoRI; S, SmaI; B, BamHI; Sa, SalI, H, HindIII; Pv, PvuI.
necessary translational signals, the insertion of promoter fragments resulted in expression of the CAT gene which was manifested as the ability of recombinant cells to grow in the presence of chloramphenicol.

A number of smaller promoter-containing fragments were isolated from the rRNA insert of pHD1.8 by making use of restriction sites predicted from the known DNA sequence (103). These were cloned into the *Sma*I site of pKK232-8 and recombinants were selected on Luria agar containing 50 ug/ml chloramphenicol. Only clones containing a functional promoter in the proper orientation were selected by this protocol. The presence of cloned inserts could be verified by isolating plasmid DNA and digesting with *EcoR1* and *BamHI* to liberate the insert (see for example Fig. 4). A schematic representation of the various *rrnB* promoter clones isolated from pHD1.8 is given in Fig. 3A. As well, the relevant DNA sequence of this region of the *B. subtilis* *rrnB* operon is shown in Fig. 3B. Note that throughout this work the nomenclature of plasmids is as follows: KK refers to a CAT-based vector, the number corresponds to the size of the insert in base pairs, and Ec or B indicate whether the insert was derived from *E. coli* or *B. subtilis* respectively. The plasmid pKK-427B was derived by digesting the pHD1.8 insert with *DdeI* and *Sau96I* (positions 700 to 1127, Fig.3B), isolating a 427 bp fragment containing the tandem rRNA promoters and cloning this into pKK232-8. This 427 bp fragment included 218 bp of DNA upstream of the
Figure 3. Origin and sequence of B. subtilis promoter fragments.

A - A schematic diagram of the B. subtilis rRN B promoter fragments. The top line represents the 2.9 Kb EcoRI fragments from the rRN B operon cloned in pKM-1 to give pGS227 (see ref. 103). The middle line shows the 1.9 Kb EcoRI fragment subcloned in pKM-1 to give pHDI.8. The -35, -10 regions of the tandem promoters are indicated by small filled boxes. The startpoint and direction of transcription from P1 and P2 are indicated by arrows. The double line represents the 16S RNA coding region. Numbering corresponds to that given by Stewart and Bott (103) where 1 is the first base of the 5' EcoRI site. The lower part of the figure represents the various promoter fragments cloned into pKK232-8 as described in the text.

B - Base sequence of the B. subtilis rRN B promoter region. Sequence as determined by Stewart and Bott (103). The -35 and -10 regions of P1 and P2 are underlined and the startpoint of transcription is indicated by arrows. The HincII site at position 982 was used to isolate the single P1 promoter on pKK-282B and the endpoints of the pKK-285B and pKK-220B deletion fragments are indicated at positions 842 and 907 respectively. The DraI sites at positions 879 and 1090 were used to construct pKK-211B while the Sau96I site at position 1127 represents the 3' endpoint of pKK-427B, pKK-220B, and pKK-285B. The sequence around P1 marked "stringent" has homology to the region which may be involved in stringent control and the sequence marked "stem" is part of a post-transcriptional RNaseIII processing site (see text).
P1 transcription start site and 112 bp downstream of the P2 initiation site. Some of these upstream sequences were deleted by digesting pKK-427B with EcoRI, treating with Bal-31 exonuclease, and releasing the shortened promoter fragments with BamH1. These deleted fragments were isolated and cloned into the SmaI site of pKK232-8 as before. By sequencing various chloramphenicol-resistant clones (G.B. Spiegelman, personal communication) one was found which retained only the tandem P1-P2 promoters as well as the A-T rich sequence immediately upstream of P1. This clone, pKK-285B, therefore had the same 3' sequences as pKK-427B but only retained 76 bp of DNA 5' to the P1 promoter (Fig. 3A,B). Finally, the plasmid pKK-211B also carried the tandem rRNA promoters (as a 211 bp DraI fragment from positions 879 to 1090, Fig. 3A,B) but here sequences both 5' and 3' to the promoter had been removed. This fragment retained only 45 bp upstream of P1 and 71 bp downstream of P2 (Fig. 3B).

The tandem P1-P2 promoters were also separated and individually cloned onto pKK232-8, again by selecting for functional expression as indicated by the appearance of chloramphenicol resistance. Initially, the 427 bp insert from pKK-427B was digested with HindII and a 282 bp fragment whose 3' terminus ended in the -35 region of the P2 promoter was isolated and subcloned. The resultant plasmid (pKK-282B, Fig. 3A,B) contained only a functional P1 promoter. A derivative containing
Figure 4. Promoter inserts in pKK232-8 and their electrophoretic mobility.

Top - Lanes A and B, pKK427B digested with EcoRI and BamHI; Lane C, pKK-220B digested with EcoRI and BamHI; Lane D, pKK232-8 vector digested with EcoRI and BamHI; Lane E, 029 digested with HindIII as molecular weight markers. Sizes of the marker fragments, in base pairs, are shown at right. Digests were run on 4% polyacrylamide gels in Tris-borate-EDTA buffer. Note the presence of the 427 bp (P1-P2), and 220 bp (P2) promoter inserts in pKK-427B and pKK-220B. The actual size of these fragments is 437 bp and 230 bp because cloning into the SmaI site of pKK232-8 and subsequent liberation of the fragment with EcoRI and BamHI results in the addition of 10 extra base pairs derived from the multiple cloning site polylinker of pKK232-8. The remaining two bands of 300 bp and 180 bp in Lanes A - D are derived from the pKK232-8 vector.

Bottom - A semilogarithmic plot of gel mobility vs. the fragment length based on the above figure. The solid dots are the molecular weight markers from 170 bp to 1091 bp. The cross represents the 427 bp promoter fragment (taken as 437 bp for measurement purposes) and the open square is the 220 bp (or 230 bp) promoter fragment.
MIGRATION DISTANCE (mm.)

FRAGMENT LENGTH (bp x 100)
only the P2 promoter was obtained by sequencing a set of Bal-31 generated deletion fragments as described above. One was found (pKK-220B, Fig. 3A,B) in which the -35 region of P1 had been deleted, thereby inactivating this promoter and leaving only the downstream P2 promoter intact. The electrophoretic mobility of some of these *B. subtilis* promoter inserts relative to DNA fragments of known size was determined and is graphically represented in Fig. 4. As can be seen, the actual size of these fragments, as judged by polyacrylamide gel electrophoresis, is very close to that calculated from the known DNA sequence. This indicates that these fragments do not display an anomalous electrophoretic behaviour.

Because the objective was to be able to compare the *in vivo* activities of *B. subtilis* and *E. coli* rRNA promoters, the analogous promoter fragments from the *E. coli* *rrnB* operon were also isolated and cloned onto pKK232-8. The plasmid pKK10-2, constructed by Brosius (132), contained a 1600 bp fragment of DNA derived from the *E. coli* *rrnB* operon. pKK10-2 was digested with *TaqI* and a 292bp fragment containing the tandem P1-P2 promoters was isolated. This fragment contains 101 bp of DNA upstream of P1 and 68 bp downstream of P2 (see ref. 133 and Fig. 5 for sequence). Insertion of this *TaqI* fragment into pKK232-8 yielded pKK-292Ec. Secondly, the *E. coli* *rrnB* P1 promoter was isolated as a 351 bp *HinfI* fragment from pKK10-2 to give pKK-351Ec. The *HinfI* restriction sites were located 256 bp upstream of P1 and
Figure 5. Base sequence of the *E. coli* *rrnB* promoter region.

Sequence as determined by Brosius et al. (21). The -35, -10 region of P1 and P2 are overlined and the startpoints and direction of transcription are indicated by arrows. Box B, A, and C refer to sequences thought to be required for antitermination of rRNA transcripts (see text). Restriction sites indicated were used in the isolation of promoter fragments for cloning into pKK232-8 as described in the text.
within the -35 region of P2 (133); thus only the *E. coli* P1 promoter was functional on pKK-351Ec and represented a construct exactly analogous to pKK-282B described above. The *E. coli* P2 promoter was cloned as a 128 bp *HhaI* fragment from pKK10-2 to give pKK-128Ec. The 5' end of this fragment was located immediately downstream of the -10 region of P1 while the 3' end was 3 bp downstream of the transcription initiation site of P2 (133, Fig. 5).

Finally, as a control, a non-ribosomal promoter from *E. coli* was also used in these experiments. The 377 bp *EcoR1*-BamHI fragment from pBR322 which carries the promoter for the tetracycline resistance gene (134) was cloned into pKK232-8 to yield pKK-Tet. A list of all plasmids constructed as CAT gene fusions is given in Table 2.
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter Insert</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK-427B</td>
<td>P1-P2</td>
<td>E. subtilis</td>
</tr>
<tr>
<td>pKK-285B</td>
<td>P1-P2, 5' deletion</td>
<td>&quot;</td>
</tr>
<tr>
<td>pKK-211B</td>
<td>P1-P2, 5'+3' deletion</td>
<td>&quot;</td>
</tr>
<tr>
<td>pKK-282B</td>
<td>P1</td>
<td>&quot;</td>
</tr>
<tr>
<td>pKK-220B</td>
<td>P2</td>
<td>&quot;</td>
</tr>
<tr>
<td>pKK-292Ec</td>
<td>P1-P2</td>
<td>E. Coli</td>
</tr>
<tr>
<td>pKK-351Ec</td>
<td>P1</td>
<td>&quot;</td>
</tr>
<tr>
<td>pKK-128Ec</td>
<td>P2</td>
<td>&quot;</td>
</tr>
<tr>
<td>pKK-Tet</td>
<td>tetracycline</td>
<td>E. coli</td>
</tr>
</tbody>
</table>

The stability of various pKK derivatives was tested to assess the extent to which cloned promoter inserts could be lost under non-selective conditions. The pKK derivatives in Table 2 were both chloramphenicol resistant because of inserted promoters and ampicillin resistant because of the \textit{bla} gene on pKK232-8 (Fig. 2). \textit{E. coli} HB101 carrying either pKK-427B or pKK-292Ec was grown for 8 hr. in broth supplemented only with ampicillin, then diluted 1 in 500 in fresh ampicillin broth and grown for a further 18 hr. Samples from both the 8 hr. and 26 hr. cultures were then plated on media containing either ampicillin or chloramphenicol and resistant colonies were counted. The number of chloramphenicol resistant clones (due to the presence of a functional promoter insert) was expressed as a percentage of the ampicillin resistant clones present (Table 3).

As can be seen, the Cm phenotype was rapidly lost under conditions that did not directly select for the maintenance of a functional promoter insert. If however, these clones were grown continually in the presence of chloramphenicol then all cells retained a functional promoter which was indistinguishable, based on electrophoretic patterns in polyacrylamide gels, from the original cloned promoter fragment (data not shown). Thus, all growth experiments with pKK derivatives were carried out in the presence of chloramphenicol to ensure promoter stability.
**Table 3.** Stability of Cm<sup>r</sup> phenotype.

<table>
<thead>
<tr>
<th></th>
<th>0 hr.</th>
<th>8 hr.</th>
<th>26 hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKK-427B</td>
<td>100</td>
<td>61</td>
<td>0.12</td>
</tr>
<tr>
<td>pKK-292Ec</td>
<td>100</td>
<td>72</td>
<td>0.80</td>
</tr>
</tbody>
</table>
3. Characteristics of chloramphenicol acetyltransferase (CAT) assay.

The assay for CAT takes advantage of the generation of a free CoA sulfhydryl group coincident with the transfer of the acetyl group to chloramphenicol catalyzed by the CAT enzyme. Reaction of the reduced CoA with 5,5'-dithiobis-2-nitrobenzoic acid yields, among other products, a molar equivalent of free 5-thio-2-nitrobenzoate which can be measured spectrophotometrically at 412 nm (128). To show that the CAT assay was linear over a range of input enzyme concentrations, different volumes of HB101/pKK-427B cell-free extract were added to the standard assay (see Materials and Methods). As shown in Fig. 6, the CAT assay appeared to deviate slightly from linearity only at very high enzyme inputs; therefore all assays were performed using between 5 and 50 ul of cell-free extract. Chloramphenicol acetyltransferase activities were expressed as units (as defined in the Materials and Methods). Normalization to total protein rather than to cell number was used because cells grown in different media are of different size (135). Sonication was used to disrupt cells because it was known that cells grown in different media broke with different efficiencies using other breakage methods (136).
Fig. 6. Linearity of chloramphenicol acetyltransferase assay.

Chloramphenicol acetyltransferase (CAT) activity was determined as described in the Materials and Methods from different volumes of cell-free extract. Extracts were prepared from *E. coli* HB101 carrying pKK-211B. CAT activity here is shown as the increase in A\textsubscript{412} per minute. To convert this to the units of CAT activity as defined in the Materials and Methods, the A\textsubscript{412} increase per minute was divided by 13.6 (Ref. 128) and then expressed on a per milligram of total protein basis.
4. Expression of *E. coli* rrnB promoters at different growth rates.

The *E. coli* HB101 host carrying either pKK-292Ec, pKK-351Ec, or pKK-128Ec was grown in different media so as to achieve a range of growth rates, then cells were harvested and assayed for CAT activity. As seen in Fig. 7, the level of CAT specific activity due to transcription from the *E. coli* P1-P2 tandem rRNA promoters increased with a steep positive slope at increasing growth rates. This was characteristic of rRNA synthesis in general (26) showing that, as expected, these promoters were regulated in a growth rate dependent manner. Furthermore, Fig. 8 shows that only the upstream P1 promoter of this pair was growth rate regulated whereas expression of the P2 promoter was at an overall lower level and did not increase with increasing growth rate. As a control, the results for pKK-Tet (Fig. 9) indicated that this non-ribosomal promoter did not show any growth rate dependent changes and overall expression was much lower when compared to the rRNA promoters.

5. Expression of *B. subtilis* rrnB promoters in *E. coli*.

The results for the *B. subtilis* rrnB-CAT fusions, expressed in the heterologous *E. coli* HB101 host, are shown in Figures 10 to 13. The tandem rrnB P1-P2 promoters (pKK-427B, Fig. 10) showed the steep positive slope with increasing growth rate
Figure 7. CAT activity vs. growth rate: pKK-292Ec in _E.coli_ HB101.

Chloramphenicol acetyltransferase specific activity was measured as a function of cellular growth rate as described in the Materials and Methods. The slope of the line was determined by linear regression. pKK-292Ec represents the tandem P1-P2 _E.coli_ rrnB promoter.
Figure 8. CAT activity vs. growth rate: pKK-351Ec, pKK-128Ec in *E. coli* HB101.

circles, *E. coli* rrnB single upstream (P1) promoter on pKK-351Ec. triangles, *E. coli* rrnB downstream (P2) promoter on pKK-128Ec. Slopes were determined by linear regression.
Figure 9. CAT activity vs. growth rate: pKK-Tet in *E. coli* HB101.

pKK-Tet represents the pBR322 tetracycline resistance promoter cloned in pKK232-8. Slope was derived by linear regression.
indicative of a classical growth rate dependent response. The same response was seen for two other clones which differed from pKK-427B in the amount of 5' and 3' flanking DNA remaining (pKK-285B, pKK-211B; Fig. 11 and 12). This suggested that sequences at least beyond -83 relative to the P1 transcription start site and beyond +77 relative to P2 were not required to elicit the growth rate dependent response. It was noted that the slope of the line determined for pKK-211B was slightly greater than that for pKK-427B or pKK-285B. The reason for this is unclear but is probably not due to a difference in translational efficiency because of the altered 3' end of this construction (see below).

Finally, Fig. 13 illustrates the results obtained for the separated promoters of the rrnB P1-P2 pair. Here it appeared that the downstream P2 promoter (pKK-220B) was under growth rate dependent control since the level of CAT specific activity increased dramatically with increasing growth rate. In contrast, the P1 promoter (pKK-282B) was transcriptionally much less active and constant over the growth rate range tested.


As mentioned previously (see Literature Review), operon fusion systems in general can be subject to a number of artifacts
Figure 10. CAT activity vs. growth rate: pKK-427B in E.coli HB101.

Chloramphenicol acetyltransferase specific activity was measured as before and slopes were calculated by linear regression. pKK-427B represents the tandem B.subtilis rrnB promoters cloned in pKK232-8.
Figure 11. CAT activity vs. growth rate: pKK-285B in *E. coli* HB101.

pKK-285B represents the P1-P2 tandem *B. subtilis* promoters with 5' flanking DNA deleted.
Figure 12. CAT activity vs. growth rate: pKK-211B in \textit{E. coli} HB101.

pKK-211B represents the P1-P2 \textit{B. subtilis} promoter with 5' and 3' flanking sequences deleted.
Figure 13. CAT activity vs. growth rate: pKK-282B, pKK-220B in E. coli HB101.

The circles represent pKK-220B (single B. subtilis P2 promoter); triangles represent pKK-282B (single B. subtilis P1 promoter).
which could potentially result in erroneous conclusions. Therefore, several experiments were carried out to ensure that the observed growth rate dependent increase in CAT specific activity was an accurate reflection of the \textit{in vivo} transcriptional activity of the cloned \textit{Bacillus} rRNA promoters.

The level of CAT messenger RNA was directly measured by hybridization of $[^3H]$ uridine labelled RNA to filter-bound M13 DNA carrying a CAT gene insert. Both orientations of the cloned insert were obtained, as shown in Fig. 14. The M13 clone carrying CAT DNA complementary to mRNA was hybridized in excess to labelled total RNA extracted from pKK-containing cells grown at different rates. The results for pKK-211B and pKK-427B (Fig. 15) show that CAT-specific mRNA increased in a growth rate dependent manner as did CAT specific activity as shown above. The ratio of CAT mRNA to CAT protein for pKK-427B, pKK-285B, and pKK-211B was essentially constant over the growth rate range tested (compare Figs. 10, 11, and 12 to Fig. 15) indicating that there were probably no differential translation effects with changing growth rate.

In addition to measuring CAT mRNA, the rate of \textit{E. coli} ribosomal RNA synthesis itself was directly measured in strains carrying pKK derivatives. Labelled RNA was extracted as before and hybridized to denatured lambda \textit{ilv5} DNA bound to nitrocellulose filters. This phage carried a single copy of a
Figure 14. Construction and orientation of M13-CAT hybridization probes.

A - 0.7% agarose gel with 2 individual M13-CAT clones. Lanes 1 and 3, M13-CAT-4; Lanes 2 and 4, M13-CAT-5. Recombinant M13 DNA is the heavy band near the top of the gel. This gel was blotted onto nitrocellulose paper which was then cut to separate lanes 1 and 2 from lanes 3 and 4.

B - autoradiograph of Southern blot in Fig. 14A after hybridization with probe DNA. Probe DNA was prepared by digesting pKK232-8 with EcoRI, labelling the 3' recessed end with α32P-dATP, redigesting with HindIII and isolating the large vector portion away from the 300 bp EcoRI-HindIII fragment (see Fig.2). The vector portion and the 300 bp EcoRI-HindIII fragment were thus each labelled only at one end. Lanes 1 and 2 were probed with the labelled vector DNA while lanes 3 and 4 were probed with the 300 bp fragment. The results indicate that M13-CAT-4 and -5 represent clones carrying the CAT gene in two different orientations with M13-CAT-5 being in the orientation complementary to CAT mRNA.
Figure 15. Amount of CAT mRNA vs. growth rate.

CAT mRNA was measured by filter hybridization to single stranded M13-CAT-5 DNA. The amount of CAT mRNA is expressed as a percentage of the total input radioactivity which bound to CAT DNA filters, after correction for background. The circles represent values obtained for pKK-427B and the squares are values for pKK-211B. The slope of the line was obtained by linear regression.
complete E. coli rRNA transcriptional unit (137). The result (Fig. 16) indicates that, as expected, the rate of rRNA synthesis increased with growth rate and suggested that the presence in these cells of multicopy plasmids carrying isolated rRNA promoters was not disruptive to overall rRNA synthesis or to the cellular mechanisms governing growth rate dependent regulation.

It had also been shown that the half-lives of some messenger RNA's could vary greatly as cellular growth rate changed (138). To eliminate the possibility that an increase in mRNA stability at high growth rates was responsible for the increase in CAT message seen above, the chemical and functional half-life of CAT mRNA was measured at high and low growth rates. The functional half-life was assessed by labelling total cellular 35S protein with [35S]methionine at various times after inhibition of transcription with rifampicin. As seen in Fig. 17 there did not appear to be a large difference in the rate of decay of CAT mRNA at high and low growth rates, with half-lives in both conditions being less that 2 min. A more accurate estimate of CAT mRNA half-life was obtained by monitoring the decay of hybridizable CAT mRNA following inhibition of transcription by rifampicin. The results in Fig. 18 indicate that the CAT mRNA from pKK-427B had a chemical half-life of approximately 43 sec. in slow growing cells and 30 sec. in faster growing cells. Thus, CAT-mRNA was slightly more labile under faster growth conditions suggesting
Figure 16. Measurement of total ribosomal RNA vs. growth rate.

_E.coli_ HB101/pKK-427B was grown with a growth rate of 1.1 doublings per hr. Total radiolabelled RNA was extracted and hybridized to filter bound lambda _ilv5_ DNA under conditions of DNA excess. The amount of rRNA is expressed as a percentage of the total input radioactivity which bound to lambda _ilv5_ filters, after correction for background. The slope of the line was determined by linear regression.
Figure 17. Determination of CAT mRNA functional half-life: CAT protein analysis.

**Top** - Total cellular protein electrophoresed through a 12-20% SDS-polyacrylamide gradient gel and stained with Coomassie Brilliant Blue R. Lane 1, *E. coli* HB101/pKK-427B grown as a stationary overnight culture; Lane 2, *E. coli* HB101/pKK-427B grown under aeration and harvested when in logarithmic growth ($A_{660} = 0.6$); Lane 3, *E. coli* HB101/pKK232-8, stationary overnight culture; Lane 4, *E. coli* HB101/pKK232-8, logarithmic culture; Lane 5, standard molecular weight markers. Sizes are noted at right, in kilodaltons. The position of the CAT protein in the strains carrying promoter-CAT fusions (Lanes 1 and 2) is indicated by the large arrow. Note that strains carrying plasmids without a promoter insert (Lanes 3 and 4) do not show detectable CAT protein.

**Bottom** - Time course of functional messenger RNA decay after arrest of transcription. *E. coli* HB101 carrying pKK-427B was grown at two different rates and total cellular proteins were radiolabelled at various times after transcription was inhibited by rifampicin (see Materials and Methods). Proteins were electrophoresed through an 18% SDS-polyacrylamide gel and autoradiographed. A, fast growth conditions (1.1 doublings per hr.). Lane 1, *E. coli* HB101/pKK232-8 (negative control); Lane 2, *E. coli* HB101/pKK427B, 0 sec. after rifampicin addition; Lanes 3 - 6, 2, 4, 7, 10 min. after rifampicin addition, respectively. B, slow growth conditions (0.50 doublings per hr.). Lane 1, *E. coli* HB101/pKK-427B, 0 sec. after rifampicin addition; Lanes 2 - 5, 2, 4, 7, and 10 min. after rifampicin addition; Lane 6, *E. coli* HB101/pKK232-8 (negative control). Note that under both fast and slow growth conditions, very little CAT protein is synthesized beyond 2 min. after inhibition of mRNA transcription.
that the data in Fig. 10 may tend to underestimate the growth rate dependent response of the *B. subtilis* rrnB promoters.

Finally, since pKK232-8 is a multicopy plasmid (131), the possibility that extreme fluctuation in plasmid copy number with varying growth rate could mimic a growth rate dependent response was considered. Others have found that growth conditions and strengths of inserted promoters could significantly affect the plasmid copy number (124, 139). Therefore, an estimate of the amount of plasmid DNA per unit of total cellular protein was obtained for several clones using a dot-blot hybridization assay. An example of such a hybridization assay is shown in Fig. 19A. Plotting the counts per minute obtained from a known input of DNA (standard curve - Fig. 19B) gave a straight line relationship at all concentrations, indicating that the amount of radiolabelled probe DNA added to the hybridization reaction was in excess of that required to hybridize to all available filter-bound DNA. The results for pKK-427B (Fig. 20) indicate that while there was a slight overall increase in the amount of plasmid DNA as growth rate increased, this was not large enough to account for the increase seen in CAT mRNA or CAT specific activity shown earlier. Measurements for other pKK clones gave similar results (data not shown).

**Discussion**
Figure 18. Chemical half-life of CAT mRNA vs. growth rate.

The chemical half-life of CAT mRNA was measured by hybridization to filter bound M13-CAT-5 DNA as described in the Materials and Methods. The results are expressed as the percentage of total input radioactivity which bound to CAT DNA filters at different times after inhibition of transcription by rifampicin. Values are for pKK-427B. The closed squares (solid line) represent cells grown at fast rates (0.97 doublings per hr.). The open squares (dashed line) represent slower growing cells (0.55 doublings per hr.). The calculated mRNA half-lives are 30 sec. and 43 sec., respectively.
Figure 19. Plasmid copy number determination by dot-blot hybridization analysis.

A - Crude cell-free extracts of *E. coli* HB101 carrying various pKK derivatives were blotted onto nitrocellulose paper using a dot-blot apparatus. In row A, a volume of extract equivalent to 10 ug of total protein was used in each case. Each spot represents a different sample of cell-free extract, derived from cells grown at different rates, and are shown for illustrative purposes only. In row B, different amounts of purified pKK232-8 DNA were blotted. Row B Lane 1, 0 ng; Lane 2, 0.1 ng; Lane 3, 0.2 ng; Lane 4, 0.4 ng; Lane 5, 0.6 ng; Lane 6, 0.8 ng; Lane 7, 1.0 ng; Lane 8, 1.2 ng; Lane 9, 1.4 ng; Lane 10, 0 ng. The filter was hybridized to an excess of labelled pKK232-8 DNA and autoradiographed.

B - Standard plasmid curve. Individual dots corresponding to row B, Lanes 1 to 9 were punched out and counted for Cherenkov radiation. Counts per minute are plotted versus the amount of pKK232-8 DNA loaded per lane. The straight line relationship indicated that the probe DNA was in excess.
COUNTS PER MIN. \((x10^3)\)

PLASMID DNA (ng.)

1.6

1.2

0.8

0.4

0
Figure 20. Amount of plasmid DNA vs. growth rate.

Values are for pKK-427B. The results were interpolated from the standard plasmid DNA curve shown in Fig. 19B. Results are expressed as the amount of plasmid DNA per microgram of total protein in the cell-free extract.
In this section it has been demonstrated that the promoters for the \textit{B. subtilis} \textit{rrnB} ribosomal RNA operon can efficiently initiate transcription of a fused gene when placed in a heterologous \textit{E. coli} host. When compared to the native \textit{E. coli} rRNA promoters both differences and similarities in the pattern of regulation as a function of changing growth rate could be discerned. The experimental approach taken here involved the construction of transcriptional fusion systems such that the promoter of interest directed the transcription of a fused, promoterless gene whose product could be easily and quantitatively assayed. This approach has proven valuable in the analysis of a variety of promoters whose native gene products were difficult or inconvenient to assay (see ref. 67 for review). In the case of ribosomal RNA operons, the rRNA product could potentially be directly measured in \textit{Bacillus} by RNA-DNA hybridization but this approach would be time-consuming and would measure only global rRNA synthesis rather than the expression from a given individual operon. As will be discussed below, the alternative approach (i.e. the study of cloned rRNA promoters as part of an operon fusion system) is both a convenient and valid means of addressing this problem.

In this work, two analogous sets of promoter fusions were constructed. In the first, the tandem (P1-P2) promoters, as well as the individual separated P1 and P2 promoters from the \textit{E. coli}
The \textit{rrnB} operon were cloned into the expression vector pKK232-8, and in the second, the P1-P2, P1, and P2 promoters from the \textit{B. subtilis} \textit{rrnB} operon were similarly cloned. The vector pKK232-8 was chosen for a number of reasons. It was specifically constructed to facilitate the cloning of unusually strong promoters by incorporating efficient transcription termination signals 3' to the fused gene (131). Thus, the stability problems normally associated with attempts to clone rRNA promoters should be overcome. Furthermore, the presence of cloned promoters could be easily detected by activation of the chloramphenicol acetyltransferase gene and this enzyme could be quantitatively assayed. Finally, direct selective pressure could be placed on all cells to ensure maintenance of the cloned promoter simply by supplementing the growth media with chloramphenicol. Using pKK232-8 therefore, a number of promoter fragments derived from the \textit{B. subtilis} \textit{rrnB} operon have been successfully cloned. In addition, problems with insert stability were eliminated as long as chloramphenicol selective pressure was maintained.

**Regulation of \textit{E. coli} \textit{rrnB} promoters.**

When cells carrying the \textit{E. coli} \textit{rrnB} - CAT fusions were grown at different rates, the level of CAT enzyme increased in a growth rate dependent manner. Conversely, CAT gene expression directed by the constitutive tetracycline-resistance gene promoter was essentially constant at all growth rates.
Furthermore, the growth rate dependent response was seen only when the tandem \textit{rrnB} P1-P2 promoters or the single upstream P1 promoter was fused to the CAT gene. These results indicate that the P1 promoter of the \textit{rrnB} operon appears to be solely responsible for the growth rate dependent \textit{in vivo} synthesis of rRNA, as shown previously by a number of workers (31, 73, 74). The downstream P2 promoter on the other hand, showed markedly reduced expression at all growth rates with no overall change as growth rate increased. The exact role of P2 remains unclear, although Sarmientos \textit{et al.} (74) have suggested that it could be responsible for the bulk of rRNA synthesis during conditions of very slow growth.

Interestingly, some of the data shown here appear to be at variance with that reported by Gourse \textit{et al.} (31). They have shown that the activity of a construct containing both P1 and P2 was higher at a low growth rate than the activity of a construct containing only P1 at the same growth rate; therefore the slope of activity vs. growth rate for the P1-P2 construct was not as steep as that of the P1 construct alone. In the data presented here however, the slope of the activity vs. growth rate plot for the P1-P2 fusion (pKK-292Ec) is greater than the slope of the P1 fusion alone (pKK-351Ec). A comparison of Figures 7 and 8 for example, shows that the activity of the P1-P2 fusion at a growth rate of 0.5 doublings per hr. was about one-half that of the P1 fusion at the same growth rate. At high growth rates however,
the situation was reversed, with the P1 fusion giving slightly less activity than the tandem P1-P2 fusion. In fact, at high growth rates, the activities of P1 and P2 appeared to be additive, i.e. the addition of the P1 activity to the P2 activity in Fig. 8 gives a level comparable to that seen for P1-P2 in Fig. 7 at high growth rates.

The reason for the discrepancy between these data and that of Gourse et al. (31) is not clear although there are several possibilities. In their study, Gourse et al. compared the expression of the isolated P1 and P2 promoters from the E. coli rrnB operon to that of the tandem P1-P2 promoters from rrnE; it is possible therefore that subtle regulatory differences exist between the seven rRNA operons in E. coli, although there is no precedent to suggest that this is so. Alternatively, the fusions used here were based on multicopy plasmids while Gourse et al. constructed single copy lysogens based on lambda phage. Although it has been shown here that plasmid copy number did not vary significantly with growth rate (see below) there may be other differences between these two systems which influence how cloned genes are expressed. It is difficult to fully assess the validity of the data of Gourse et al. since they did not report mRNA half-life changes or mRNA translational efficiencies at different growth rates from their lambda-based expression system.
The data presented here imply that the expression of the *E. coli* P1 promoter is inhibited at low growth rates by the presence of the P2 promoter in the tandem P1-P2 arrangement. When the two promoters were separated, the inhibition of P1 was released and expression increased about 2-fold at lower growth rates. At high growth rates however, no such inhibitory effects were observed. From the perspective of global rRNA regulation, this type of mechanism would seem to be advantageous since at low growth rates very little rRNA synthesis is required; therefore the P2 promoter (or sequences in proximity to it) would act by inhibiting transcription from the major growth rate regulated promoter (P1) while at the same time directing transcription of the rRNA genes so as to maintain a basal level of rRNA synthesis. When environmental conditions signal the need for more rRNA synthesis, the inhibition of P1 could be released and rRNA could be synthesized in a growth rate dependent manner until at high growth rates maximal synthesis is achieved by the combined expression of P1 plus P2. The finding of Sarmientos and Cashel (74) that cells recovering from stationary phase show a burst of P2 transcriptional activity followed by a slower increase in P1 expression does not necessarily contradict this model since the above discussion is based on slowly growing, not stationary cells, and the physiology of a culture in stationary phase is at best only poorly understood. Interestingly, the results obtained for the cloned *E. subtilis* rRNA promoters showed a similar
overall trend to that seen for the *E. coli* rRNA promoters, although the relative roles of P1 vs. P2 were reversed (see below).

**Regulation of *B. subtilis* rrnB promoters in *E. coli*.**

A 1.9 Kb fragment derived from the 5' end of the *B. subtilis* rrnB operon was initially cloned into pKM-1, a *galK*-based expression vector. Although this fragment was oriented so as to direct transcription into the *galK* gene, no obvious *galK* phenotype could be detected. The reason for the lack of *galK* expression is not clear, but, assuming that the *B. subtilis* rrnB promoters are transcriptionally active in this host, it could reflect the presence of latent transcription termination sites within the 16S RNA coding sequence present on this fragment. By analogy, Li et al. (44) have shown that sequences within the *E. coli* rrnB 16S rRNA coding region can act as efficient transcription terminators if cloned such that they are in reverse to their normal wild-type orientation.

That the *B. subtilis* promoters on this cloned 1.9 Kb fragment are still recognized by *Bacillus* RNA polymerase has been shown by V. Webb (Ph.D. thesis, University of British Columbia, 1986) who used pHD1.8 as well as the separated promoter clones as templates for *in vitro* transcription reactions. Thus, the cloning and transfer of this portion of the *B. subtilis* rrnB operon to
E. coli has probably not altered the promoters such that they are no longer functional in B. subtilis.

Using the cloned insert on pHD1.8 as a source of DNA fragments, smaller rrnB promoter fragments were isolated and cloned onto the expression vector, pKK232-8. The largest of these fragments (pKK-427B) still retained 220 bp of DNA upstream of the P1 transcriptional start site and 114 bp downstream of P2. In two other clones (pKK-285B, pKK-211B), much of the 5' flanking DNA was removed by Bal-31 digestion or by restriction endonuclease treatment and in one case only a functional P2 promoter remained (pKK-220B). Furthermore, three of these promoter clones maintained the same 3' flanking sequences (only pKK-211B and pKK-282B did not). Alterations within the transcribed but non-translated region of an mRNA transcript have in some cases been shown to influence the stability of the message (138). In the studies discussed here though, any effect that the 3' region might have on CAT gene expression should be consistent in three of the operon fusions and not influence the comparisons drawn between various constructions. Finally, as seen in Table 3, CAT fusion plasmids containing B. subtilis promoters appeared to be stable in E. coli in the presence of a direct selective pressure. Unlike other expression systems (67, 129) it can reasonably be assumed here that all cells in the population carry a functional and intact promoter insert as
long as chloramphenicol selection is maintained.

The fact that *B. subtilis* rRNA promoters could be cloned into pKK232-8 by selecting for the chloramphenicol-resistance phenotype indicates that these promoters are transcriptionally active in *E. coli*. This is not unexpected since an examination of the DNA sequence of the P1 and P2 promoter regions reveals a strong homology to the consensus sequence for *E. coli* promoters (Fig. 3B, ref. 17). When the *B. subtilis* (P1-P2) - CAT fusions were tested in *E. coli* as a function of increasing growth rate, a steep increase in CAT specific activity was seen, indicative of classical growth rate dependent expression (Figs. 10, 11, 12). However, an operon fusion system such as used here represents in some ways an artificial system since the translation product from a multicopy plasmid was measured whereas in the native state any given rRNA operon exists as a single copy per genome and produces a non-translated RNA product. Therefore several experiments were performed to ensure that the observed growth rate dependent increase in CAT specific activity was a true reflection of the *in vivo* transcriptional activity of the cloned *B. subtilis* promoters.

Firstly, to ensure that the amount of CAT enzyme produced accurately reflected the degree of synthesis of CAT mRNA, the amount of CAT mRNA was measured directly at different growth rates by hybridization to a CAT-specific probe. The results for
three clones, pKK-427B, pKK-285B, and pKK-211B, showed that CAT mRNA approximately paralleled the increase in CAT protein as a function of growth rate (compare Figs. 10, 11, and 12 to Fig. 15). The essentially constant ratios of CAT protein to CAT mRNA in these examples would imply that there were no great differences in translational efficiency which could possibly be due to the altered 3' end of the pKK-211B cloned fragment. It was therefore shown that measurements of CAT specific activity although indirect, is a valid means of assessing the transcriptional activity of promoters cloned into pKK232-8. Furthermore, the presence in cells of multiple copies of a cloned rRNA promoter does not appear to limit the growth rate dependent synthesis of rRNA in general, since the synthesis of total rRNA, as measured by hybridization to a lambda probe carrying a complete E. coli rRNA operon, followed the expected pattern (Fig. 16). This would indicate that any ancillary factors that may be required to elicit the growth rate dependent response are not titrated out by the increased number of rRNA promoters per cell.

Secondly, it has been shown that some mRNA's have significantly different half-lives at different cellular growth rates (138). The functional half-life of CAT mRNA was therefore assessed by examining the amount of CAT-protein synthesized from pKK-427B after mRNA synthesis had been inhibited. No differences were apparent between fast and slow growing cells (Fig. 17), implying that the half-life of CAT mRNA was similar (less than 2
and that the translational efficiency of the pKK-427B CAT mRNA was not altered because of differences in growth rate. A more accurate estimate of the CAT mRNA chemical half-life was obtained by hybridization analysis (Fig. 18) and it was found that the CAT message from pKK-427B was in fact slightly less stable at higher growth rates. This suggests that the CAT specific activity as seen in Fig. 10 may in reality be even greater at higher growth rate, thereby accentuating the apparent growth rate dependent response.

Finally, the relative plasmid copy number of the various constructions grown at different rates has been estimated since other workers have found that growth conditions and strengths of inserted promoters can significantly affect this parameter (124, 139). It was found that while plasmid copy number does change with growth rate, the overall trend is only a slight increase at higher growth rates, which is not enough to account for the large increase in CAT protein or CAT mRNA seen for the growth rate regulated constructions.

The fact that pKK-427B was shown both by assay of CAT protein and CAT mRNA to be growth rate regulated in *E. coli* in itself supports the suggestion that the overall control of rRNA synthesis in *Bacillus* and *E. coli* is similar (109), since the features which govern growth rate dependency, either at the DNA sequence level or any required ancillary factors, appear to be
compatible in both organisms. It is likely therefore that the basic regulatory mechanisms governing the synthesis of such key components of the cellular translational apparatus are highly conserved from an evolutionary standpoint. Interestingly, the level of activity from the Bacillus rRNA promoters was at least as high, if not greater than that seen for the analogous E. coli promoters (compare pKK-427B and pKK-292Ec, Fig. 10 and 7). The reason for this is unclear but may reflect subtle differences in the DNA sequence of the -10 and -35 regions. The -35 region of the B. subtilis P2 promoter for instance, is more homologous to the E. coli "consensus" sequence (17) than are the analogous regions of either P1 or P2 from the E. coli rrnB operon. As well, the B. subtilis promoters seem to have a more optimal spacing between the -10 and -35 regions. Whether these differences alone are enough to confer a greater overall level of activity upon the B. subtilis promoters is unknown.

It is clear that there are also differences in the overall level of expression (but not in the trend of growth rate dependency) when the three clones carrying the B. subtilis tandem P1-P2 promoters are compared (Fig. 10, 11, 12). Both pKK-285B and pKK-211B reach higher maximum levels of activity than does
pKK-427B. The pKK-211B construction was deleted both 5' and 3' to the tandem promoters, so the argument could be made that the alteration of the 3' end could possibly change the translational efficiency of this CAT mRNA such that higher specific enzyme activities are seen. However, the pKK-285B clone maintained the same 3' sequence as pKK-427B and was only deleted 5' to the promoter, although to a slightly lesser extent than pKK-211B. Here again, a higher absolute level of activity was seen, implying that this effect was only due to the extensive 5' deletion that occurred between pKK-427B and the other clones. The reason for this is presently unknown although the possibility exists that some, as yet unidentified, regulatory regions are found within these far upstream sequences.

For E. coli rRNA promoters, it has been shown that an A-T rich region between -51 and -88 relative to the P1 transcription initiation site can enhance the overall level of transcription from P1 by as much as 15-fold (31). Similar upstream activation sequences have been noted for other stable RNA genes (30, 32). In the case of the B. subtilis rnrB promoters, this region can be completely removed (in pKK-211B) without any decrease in transcriptional activity. Other workers have noted that DNA fragments containing these upstream activation elements from E. coli possess anomalous electrophoretic mobilities indicative of some conformational change in the DNA brought about
by the DNA sequence itself (30, 31). It has been shown here that
*B. subtilis* *rrnB* DNA fragments carrying the analogous sequences
upstream of P1 appear to have normal or only very slightly
altered mobilities in polyacrylamide gels (Fig. 4). Upstream
activation of rRNA promoters could therefore be a reflection of
certain topological features of the DNA which are seen in *E. coli*
but not in *B. subtilis* rRNA operons, and the presence of an A-T
rich region alone may not be sufficient to enhance downstream
transcription.

It is clear that the tandem *B. subtilis* *rrnB* promoters
expressed in *E. coli* show an overall growth rate dependency as do
the native *E. coli* rRNA promoters while apparently lacking an
upstream activation feature. However, more important differences
between these two systems become apparent when the expression of
the individual P1 and P2 promoter elements are examined. It was
observed here that, in contrast to the *E. coli* promoters, the *B.
subtilis* upstream P1 promoter was not growth rate regulated and
was only weakly expressed. The *B. subtilis* downstream P2 pro-
moter was the more active and growth rate regulated promoter of
this pair. While the P2 promoter fragment used here still re-
tains the -10 region of the P1 promoter, examination of the vec-
tor DNA sequences upstream of the 220 bp insert did not reveal a
potential -35 sequence. Therefore the critical region for growth
rate control of the *Bacillus rrnB* operon would appear to lie
in a 183 bp region between position 907 and 1090 (see Fig. 3B).
The level of expression of the isolated P2 promoter was almost twice as high at low growth rates compared to the activity of the tandem P1-P2 construction at the same growth rate (compare pKK-220B and pKK-427B at 0.5 doublings/hr.). At high growth rates the activity of the P1-P2 pair was greater and was approximately equal to that obtained if the activities of the individual P1 and P2 promoters were summed. The same trend was seen for the E. coli promoters except that, as noted above, the responses of P1 and P2 were reversed. These results would again tend to imply that this response was not due to some artifact within the system but could reflect some regulatory feature shared between these two rRNA operons.

Implications for growth rate control models.

The results presented above have shown that while the overall response to growth rate of a Bacillus rRNA promoter introduced into E. coli is the same as the tandem E. coli promoters, the relative response of the individual P1 and P2 promoters is reversed. Clearly this result has important implications in terms of the mechanistic details of growth rate dependent control in E. coli although the data presented here do not allow a distinction to be made between any of the current models for growth rate regulation. As discussed in the Literature Review, three general models have been proposed for
the regulation of stable RNA genes. The passive regulation model of Maaloe (79) is attractive because of its simplicity but is in conflict with several experimental observations. Current debate has therefore centered on which of the remaining models, the direct effector model of Bremer (78, 79), and Travers (58, 80) or the ribosome feedback model of Nomura (14, 77), is correct. Most evidence appears to favor some type of feedback inhibition mechanism acting directly on the promoters of rRNA operons (31) although the effector itself may not be the free ribosomes proposed by Nomura but some other compound responsive to the level of free ribosomes. Whether this compound is ppGpp or some other signal molecule remains to be seen.

The data obtained here with the pKK232-8 operon fusion system do not currently favor one model over the other although it may be possible to use this fusion system as a means of testing certain predictions made by both models. For example, introduction of an rrrB-CAT fusion plasmid into E. coli would result in an effective increase in the number of rRNA promoters per cell but not in the number of rRNA operons (since the cloned promoter fragments do not contain any rRNA sequences). This is analogous to the system described by Jinks-Robertson et al. (77) in which the introduction of extra deleted copies of rRNA operons failed to produce a decrease in the transcription of chromosomal rRNA operons. The introduction of extra complete rRNA operons on the other hand, resulted in a gene dosage dependent decrease
in overall rRNA transcription from each chromosomal operon. It would be predicted therefore, that introduction of extra rRNA promoters (i.e. \texttt{rrnB-CAT} fusion plasmids) would not lead to any changes in the level of chromosomal rRNA transcription. As can be seen in Fig. 16, the introduction of a \textit{B. subtilis} \texttt{rrnB-CAT} fusion into \textit{E. coli} does not appear to diminish the overall growth rate response of chromosomal rRNA operons although further experiments are necessary to accurately quantitate the transcriptional activity of chromosomal rRNA operons under such conditions. Additionally, one could envisage that the introduction of extra complete \textit{E. coli} rRNA operons into a host already carrying a \textit{B. subtilis} \texttt{rrnB-CAT} fusion plasmid should result in a reduction of the level of \textit{B. subtilis} rRNA promoter transcription if the ribosome feedback model was correct. Interestingly however, the sequences identified by Gourse et al. (31) as being critical for both growth rate dependent regulation and feedback inhibition of the \textit{E. coli} \texttt{rrnB} operon (-51 to -20 of P1) are not homologous to the equivalent region of the \textit{B. subtilis} \texttt{rrnB} promoter. The results of the experiments described above would therefore be difficult to predict.

Conversely, the direct effector model would predict that ppGpp would inhibit transcription from the \textit{B. subtilis} \texttt{rrnB} promoters to the extent seen for the native \textit{E. coli} rRNA promoters since both are growth rate regulated in \textit{E. coli}. This could be directly tested using the \texttt{rrnB-CAT} fusion plasmids. In
this way it may also be possible to provide some clues as to the identity of the hypothetical intermediary effector molecule which senses free ribosome levels and subsequently interacts with rRNA promoters, since this alternative to the ribosome feedback model may ultimately prove to be correct.

As mentioned earlier, the data presented in this section are significant from a mechanistic point of view and must be taken into consideration when the details of growth rate dependent regulation are established. For example, the A-T rich region immediately upstream of many rRNA and tRNA promoters, including the Bacillus P1 promoter studied here, has long been thought to play a role in modulating downstream transcription (64, 65). It was shown here that this region can be largely deleted with no change in the ability of the tandem promoters to be growth rate regulated. In fact, the pKK-220B construction (P2) which is still under growth rate dependent control, lacks this region entirely although there is a moderate A-T bias in the region immediately preceding the P2 -35 site (Fig. 3B). As mentioned above, no obvious DNA sequence homology can be seen between the region immediately surrounding the B. subtilis P2 -35 site and the same region surrounding the E. coli rrnB P1 promoter (compare Fig. 3B and 5), although this region is apparently critical for the growth rate regulated expression of E. coli P1 (31). This suggests that the control points for growth rate control and
possibly feedback regulation as well may not lie in a precise, conserved DNA sequence within the \textit{rrn} promoters, but instead are more subtle and probably reflect certain conformational or topological characteristics of rRNA promoters which in turn can be achieved through a number of different possible DNA sequences.
Chapter 2

Construction of alternative promoter-probe cloning vectors.

Introduction

In Chapter 1, the growth rate dependent expression of B. subtilis rrnB promoters in E. coli has been characterized. It was now desirable to determine whether the relative effects of growth rate on P1 vs. P2 expression seen in E. coli were true in vivo for B. subtilis as well. Unfortunately, operon fusions based on the pKK232-8 expression system were not directly transferable to B. subtilis for a number of reasons. Since pKK232-8 was based on an E. coli pBR322 replicon, it would not be expected to replicate as an autonomous plasmid in any Gram-positive organism. In general, plasmids used in Bacillus have been constructed so as to include a Gram-positive specific origin of replication (ori) region (140). Secondly, a promoter-CAT fusion on pKK232-8, even if introduced into B. subtilis, would not give rise to the CAT protein in this organism. While some genes from Gram-negative organisms could be transcribed relatively efficiently in B. subtilis (141, 143), the messenger RNA's so formed were generally not translated into protein. McLaughlin et al. (142) have determined that this translational block was due to the lack of sufficient complementarity between the ribosome binding site of Gram-negative transcripts and the
3' end of the 16S RNA of *B. subtilis*. However, a number of Gram-negative mRNA's have been found to be translated in *Bacillus* because their ribosome binding sites have, for unknown reasons, been extended such that the required homology to *B. subtilis* 16S RNA was achieved (126). An ideal vector for the purposes outlined above would therefore be one which carried dual origins of replication such that it could replicate in both *E. coli* and *B. subtilis* and, at the same time, carried a promoterless marker gene which could be fully expressed in both organisms. Thus, the required operon fusions could be constructed in *E. coli* where the plasmid manipulations were technically simpler and more efficient, followed by transfer of the fusion plasmid to *B. subtilis* where specific regulatory characteristics could be tested.

Zukowski et al. (126) have shown that the *xylE* gene from *Pseudomonas putida* was expressed and translated in *B. subtilis* and in *E. coli*. This gene was found as part of a multi-gene operon on the TOL plasmid of *Pseudomonas putida* and participated in the catabolism of toluene and related aromatic hydrocarbons through the corresponding catechols to pyruvate (145). The *xylE* gene itself encoded a catechol 2,3-dioxygenase which converted the colorless substrate catechol to the yellow-colored 2-hydroxymuconic semialdehyde product. Using the promoterless *xylE* gene, Zukowski et al. have constructed a promoter-probe vector for *B. subtilis* which replicated in *Bacillus* and in *E. coli*,.
constitutively produced dioxygenase activity in \textit{E. coli}, but only produced dioxygenase in \textit{B. subtilis} if a promoter was placed 5' to the \textit{xylE} gene. As an initial step toward the goal of constructing a dual \textit{E. coli} - \textit{B. subtilis} operon fusion system, the \textit{xylE} gene was used as the basis for a set of vectors which thus far only replicate in \textit{E. coli} but, unlike the Zukowski \textit{et al.} plasmids, can be used to identify promoter-bearing fragments in \textit{E. coli}, can stably maintain unusually strong promoters, and have the potential to be modified for direct use in \textit{B. subtilis}.

Results


A 2.0 Kb \textit{BamHI-XhoI} fragment carrying the \textit{xylE} gene (126) was isolated from the TOL plasmid, ligated to pBR322 cut with \textit{BamHI} and \textit{SalI}, and used to transform \textit{E. coli} HB101 to ampicillin resistance. Clones carrying the \textit{xylE} fragment were identified by spraying colonies with an aqueous solution of catechol as described in the Materials and Methods and selecting those colonies which turned a yellow color. Expression of the \textit{xylE} gene in this case was presumably due to transcription initiating at the \textit{tet} promoter of pBR322 (144). DNA from one of these positive clones was digested with \textit{EcoRI} and \textit{NruI} and the \textit{xylE} fragment isolated and ligated to \textit{EcoRI-NruI} digested pKK9-4.
This construction resulted in the joining of the transcription terminator from the \textit{E. coli} rrnB operon to the 3' end of the \textit{xylE} gene. Finally, the \textit{tet} promoter was eliminated by replacing the \textit{PstI-BamH1} fragment in this vector with the \textit{PstI-BamH1} fragment of pKK232-8 (131). The latter fragment contained an upstream transcription terminator and multiple cloning sites for insertion of promoter fragments. The resulting plasmid, pTLXT-11, had the structure shown in Fig. 21.

The vector pAS-3 (Fig. 21) was created by introducing the lambda tr1 transcription terminator (isolated as a \textit{HindIII-Rsal} fragment from pKM-1, ref. 129) into the unique \textit{HpaI} site of pTLXT-11. This resulted in the positioning of the lambda terminator between the promoter cloning site and the start of the \textit{xylE} structural gene, in a fashion analogous to that of pKM-1 (129).

2. Cloning and stability of promoter fragments.

Attempts were made to subclone all the \textit{B. subtilis} rrnB promoter fragments shown in Fig. 3A into pTLXT-11 or pAS-3. These fragments were isolated from the various pKK derivatives (Chapter 1, Fig. 3A), cloned into the \textit{SmaI} site of the \textit{xylE} vectors, and used to transform \textit{E. coli} HB101 to ampicillin + resistance. Clones expressing the \textit{xylE} phenotype were selected on the basis of the yellow color produced after spraying colonies
Figure 21. Structure of pTLXT-11/pAS-3.

pTLXT-11 was constructed as described in the text. The thick bar, with circles, was derived from the TOL plasmid and carries the xylE gene. The single line denotes pBR322 sequences. A multiple cloning site linker, used for insertion of promoter fragments, is positioned proximal to the xylE gene and a single Tl transcription terminator is proximal to this. The position of the downstream transcription termination signals (T1, T2), derived from the E.coli rrnB operon, is indicated. A 5S RNA gene was included during the cloning procedure. pAS-3 was constructed by inserting a 500 bp fragment carrying the lambda tRl transcription terminator (from pKM-1) into the unique HpaI site of pTLXT-11.

Restriction sites are: Ps, PstI; PvuII; Pv, PvuI; E, EcoRI; S, SmaI; B, BamHI; H, HpaI; K, KpnI; Sa, SalI; A, AvaI. Bracketted sites have been destroyed during cloning.
with a catechol solution. While all promoter fragments could initially be cloned into either \textit{xylE} vector, it was found that constructs carrying any tandem (P1-P2) promoter fragment in pTLXT-11 were highly unstable. If a single yellow colony was subcultured into ampicillin-supplemented broth and grown to saturation, from 85\% to 90\% of the cells in the resultant population were found to have lost the \textit{xylE} phenotype, although plasmid-mediated ampicillin resistance was still present. Up to 100\% of the cells in the population could become \textit{xylE} upon further subculturing. Restriction endonuclease analysis indicated that plasmids from \textit{xylE} clones had lost the entire \textit{promoter} insert. Stable \textit{xylE} clones could eventually be isolated by continually picking and re-growing single yellow colonies, but plasmids from these clones carried deleted versions of the original promoter insert. In all cases, the deletion specifically involved the 3' end of the promoter fragment (data not shown). Clones carrying either the single \textit{rrnB} P1 or P2 promoters in pTLXT-11 were stable however. Likewise, all the tandem or single promoter fragments were completely stable in pAS-3, indicating that the presence of an internal transcription termination site (in pAS-3) was sufficient to stabilize the strong tandem promoters.

Similar attempts were made to clone the \textit{E. coli} \textit{rrnB} promoter fragments described in Chapter 1. Here again, the
tandem promoter fragments were highly unstable in pTLXT-11, but interestingly, this instability was noted for pAS-3 as well. In addition, only the isolated *E. coli* P2 promoter was stable in pTLXT-11. The upstream P1 promoter could be maintained in pAS-3 but was found to be unstable when cloned into pTLXT-11.

Finally, a non-ribosomal RNA promoter was cloned as well. A 253 bp *MspI*-MnI fragment from the *Staphlococcus aureus* plasmid pC194 carrying the promoter for the chloramphenicol acetyltransferase gene (146) was cloned into pTLXT-11. Attempts to clone this fragment into pAS-3 failed, possibly because the level of *xylE* expression was lowered to the extent that positive clones could not be detected (see below). Table 4 summarizes the various operon fusions constructed as outlined above.
Table 4 - Ribosomal RNA promoter - xylE operon fusions

B.s. = B. subtilis
E.c. = E. coli

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter</th>
<th>Internal lambda terminator</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS-427B</td>
<td>B.s. P1-P2</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>pAS-211B</td>
<td>B.s. P1-P2</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>pTLXT-427B</td>
<td>B.s. P1-P2</td>
<td>-</td>
<td>no</td>
</tr>
<tr>
<td>pTLXT-211B</td>
<td>B.s. P1-P2</td>
<td>-</td>
<td>no</td>
</tr>
<tr>
<td>pAS-282B</td>
<td>B.s. P1</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>pTLXT-282B</td>
<td>B.s. P1</td>
<td>-</td>
<td>yes</td>
</tr>
<tr>
<td>pAS-220B</td>
<td>B.s. P2</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>pTLXT-220B</td>
<td>B.s. P2</td>
<td>-</td>
<td>yes</td>
</tr>
<tr>
<td>pAS-292Ec</td>
<td>E.c. P1-P2</td>
<td>+</td>
<td>no</td>
</tr>
<tr>
<td>pTLXT-292Ec</td>
<td>E.c. P1-P2</td>
<td>-</td>
<td>no</td>
</tr>
<tr>
<td>pAS-351Ec</td>
<td>E.c. P1</td>
<td>+</td>
<td>yes</td>
</tr>
<tr>
<td>pTLXT-351Ec</td>
<td>E.c. P1</td>
<td>-</td>
<td>no</td>
</tr>
<tr>
<td>pTLXT-128Ec</td>
<td>E.c. P2</td>
<td>-</td>
<td>yes</td>
</tr>
<tr>
<td>pTLXT-253</td>
<td>pC194 CAT</td>
<td>-</td>
<td>yes</td>
</tr>
</tbody>
</table>
3. Expression of promoter-xylE fusions in E. coli.

Only those strains carrying plasmids which had been shown to maintain a stable xylE phenotype (Table 4) were further studied. Cells were grown as described in the Materials and Methods so as to achieve a range of growth rates, harvested, and assayed for catechol 2,3-dioxygenase activity. Figure 22 illustrates the changes in dioxygenase specific activity as a function of growth rate for all plasmids carrying B. subtilis rRNA promoters as well as for pTLXT-253 carrying the constitutive CAT gene promoter. As can be seen, all constructions gave slightly lower levels of dioxygenase activity at higher growth rates. No growth rate dependent increase in activity was observed, even for plasmids carrying rRNA promoters which had previously been shown to be growth rate regulated in E. coli (i.e. 427 bp P1-P2, 220 bp P2 - Chapter 1). When the response of the cloned E. coli rrnB promoter as a function of growth rate was examined, a similar trend was observed. In this case, it was only possible to study the individual P1 and P2 promoters in pAS-3 and pTLXT-11 respectively, since all other clones were unstable. However, pAS-351Ec, carrying the rrnB P1 promoter which had been shown to be growth rate regulated here (Chapter 1) and elsewhere (31) did not produce a growth rate dependent increase in catechol 2,3-
Expression of the xylE gene from various B. subtilis rrnB promoter inserts was assessed by determining the specific activity of catechol 2,3 dioxygenase as described in the Materials and Methods. Growth rate was calculated as the reciprocal of the cellular doubling time in hours. The host for all plasmids in these experiments was E. coli HB101. Line 1 (open circles), pTLXT-220B; Line 2 (closed circles), pTLXT-282B; Line 3 (stars), pTLXT-253 (CAT gene promoter); Line 4 (open squares), pAS-427B; Line 5 (closed squares), pAS-220B; Line 6 (open triangles), pAS-282B. See Table 4 for a summary of the characteristics of the plasmids used here.
Figure 23. Catechol 2,3 dioxygenase activity vs. growth rate:
pAS-351Ec.

_E.coli_ HB101 carrying pAS-351Ec (E.coli rrnB P1 promoter) was
grown at different rates and catechol 2,3 dioxygenase specific
activity determined from cell-free extracts. The slope of the
line was determined by linear regression.
dioxygenase activity (Fig. 23).

4. Effects of lambda tR1 terminator on expression of *B. subtilis* *rrnB* promoters.

An examination of the data in Fig. 22 provides additional information as to the efficiency of transcription termination at the internal lambda terminator carried on pAS-3. At a growth rate of 1.0 doubling/hr., the P1 promoter on pAS-282B gave only 3.4% of the catechol 2,3-dioxygenase activity seen for P1 on pTLXT-282B (Table 5). Therefore, assuming that plasmid copy number and translational efficiencies were equivalent in both cases, this implies that over 96% of the transcripts originating at P1 were terminated at the lambda tR1 site. For the P2 promoter on pAS-220B, approximately 93% of the transcripts were terminated before reaching the *xylE* structural gene (Table 5).

Finally, it was noted that the relative activities of the *B. subtilis* P1 and P2 promoters followed the same trend as observed earlier using the pKK232-8 expression system. The P2 promoter gave higher levels of catechol 2,3-dioxygenase activity at all growth rates in both pTLXT-11 and pAS-3. At a growth rate of 1.0 for example, P2 resulted in approximately 50% more activity than P1 in the pTLXT-11 and 76% more activity in the pAS-3 vector (Table 5).
Table 5 - Activities of *B. subtilis* promoters in *E. coli*

(u = 1.0)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Promoter</th>
<th>Internal lambda terminator</th>
<th>Catechol 2,3-dioxygenase (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS-427B</td>
<td>P1-P2</td>
<td>+</td>
<td>2,800</td>
</tr>
<tr>
<td>pAS-282B</td>
<td>P1</td>
<td>+</td>
<td>340</td>
</tr>
<tr>
<td>pTLXT-282B</td>
<td>P1</td>
<td>-</td>
<td>10,100</td>
</tr>
<tr>
<td>pAS-220B</td>
<td>P2</td>
<td>+</td>
<td>1,450</td>
</tr>
<tr>
<td>pTLXT-220B</td>
<td>P2</td>
<td>-</td>
<td>20,050</td>
</tr>
<tr>
<td>pTLXT-253</td>
<td>CAT</td>
<td>-</td>
<td>3,250</td>
</tr>
</tbody>
</table>
Discussion

This chapter described the construction of two promoter-probe vectors, pTLXT-11 and pAS-3, and their applicability in the cloning of strong ribosomal RNA promoters from B. subtilis and E. coli. These vectors used the promoterless Pseudomonas putida xylE gene encoding a catechol 2,3-dioxygenase enzyme which could be quantitatively assayed and had the added advantage that the xylE gene itself could potentially be expressed in B. subtilis without further modifications (126). Functionally, pTLXT-11 was identical to pKK232-8 described by Brosius (131) although structurally they differed in that pTLXT-11 used the xylE gene whereas pKK232-8 contained a promoterless CAT gene. Therefore, promoters cloned into pTLXT-11 could not be placed under constant selective pressure as they could in the pKK232-8 system. Also, pTLXT-11 was similar to pTG402, another xylE based promoter-probe plasmid constructed by Zukowski et al. (126). Unlike pTG402 however, pTLXT-11 could not replicate in B. subtilis and did not constitutively express the xylE gene in E. coli because all upstream pBR322-derived promoters had been eliminated.

It was expected that as with pKK232-8, pTLXT-11 could be used to clone and stably maintain all types of strong promoters such as those of the rRNA operons. It was found however, that the tandem rRNA promoters from B. subtilis could be cloned by
selecting for the $xyle^+$ phenotype, but that $xyle^-$ segregants rapidly appeared following outgrowth in ampicillin-supplemented media. Stable $xyle^+$ clones could eventually be obtained but preliminary characterization of their promoter inserts revealed that extensive deletions involving the 3' ends had occurred. This suggested that these stable clones could have lost the P2 element of the promoter insert. This instability of tandem promoter fragments should not be due to read-through transcription from the rRNA promoters into the plasmid origin of replication since a double set of efficient rRNA transcription terminators were located downstream of the $xyle$ gene. It is possible that the instability was due to the detrimental effects of a hybrid $rrnB-xyle$ mRNA species in $E. coli$ or that this mRNA was so abundant that it placed a drain on the cellular protein synthesis machinery, namely the ribosome and tRNA pools. This possibility is argued against however, by the finding that these same promoter fragments were stable in pKK232-8 (Chapter 1) which also produces a hybrid mRNA and which, assuming equivalent translation efficiencies, must place the same demand on the protein synthetic capacity of the cell. Instead the results presented here tend to imply that the product of the $xyle$ gene may be toxic to $E. coli$ when produced at the high levels expected for transcription initiating at rRNA promoters. While this has not been directly proven here, several other workers have found that overexpression of certain plasmid-encoded proteins were detrimental to cellular metabolism and the stability
of recombinant plasmids. Brosius (132) for example, found that overexpression of the rat insulin gene on a multicopy plasmid led to plasmid instability and cellular lysis; an effect not due to overproduction of the mRNA itself and not due to an inability of the cell to efficiently export the insulin protein since overproduction of the non-secreted form of the protein was still lethal to the cell. In the case of the xylE fusions, the high level of catechol 2,3-dioxygenase enzyme may in some way be detrimental or inhibitory to a required cellular metabolic function. This possibility has not been rigorously tested however, so other factors could still be important for the observed instability. Nevertheless, it was found that the tandem \textit{Bacillus} rRNA promoters could be stably maintained on xylE vectors simply by the placement of a lambda tR1 terminator between the promoters and the xylE structural gene (pAS-3). The presence of this terminator reduced catechol 2,3-dioxygenase activity by 93 to 97\% and presumably would bring the amount of dioxygenase protein down to a level tolerated by \textit{E. coli}.

Interestingly, it was found that the tandem \textit{rrnB} promoters from \textit{E. coli} could not be stably maintained even in the terminator-containing pAS-3 vector. It had been shown in a number of studies that RNA polymerase initiating at \textit{E. coli} rRNA promoters could efficiently read-through rho-factor dependent termination signals as a means of overcoming the transcriptional
polarity normally associated with non-translated operons (37, 38, 44). This antitermination mechanism was dependent on DNA sequences located less than 67 bp distal to the P2 promoter element (31), sequences which were present on the rRNA fragment cloned on pAS-292Ec. It is possible, although not rigorously proven, that the inability to maintain the 292 bp rrnB promoter fragment was due to the antitermination properties conferred by these sequences which resulted in read-through transcription of the rho-dependent lambda tR1 terminator on pAS-3. In contrast, the 427 bp tandem rRNA promoter fragment from B. subtilis, which included 114 bp of DNA distal to the P2 element, was completely stable in pAS-3. This suggested that the lambda tR1 terminator in pAS-3 was functional in preventing a large percentage of read-through transcription and implied that either the B. subtilis rrnB promoters had no antitermination mechanism analogous to that of the E. coli promoters, or else one was present but was i) not functional in E. coli or, ii) inoperable on the particular rho-dependent terminator used here or, iii) located beyond the 3' end of the 427 bp promoter fragment. The data presented here do not allow one to distinguish between these possibilities. An examination of the DNA sequence distal to the B. subtilis rrnB P2 promoter up to the start of the 16S RNA gene failed to reveal a region homologous to the so-called Box B, A, C region thought to to be required in the antitermination mechanism in E. coli (31, 44). Similarly, no such region appeared in the intervening sequence between the B. subtilis P1 and P2 promoters.
Unfortunately, the mechanisms of transcription termination and transcription-translation coupling in *B. subtilis* are poorly understood. While a protein similar to the *E. coli* rho factor has been reported in *Bacillus* (147), a rigorous genetic proof of the existence of such a factor (i.e. as a mutation able to suppress polar mutations) has not yet appeared. It is therefore possible that *B. subtilis* rRNA operons may not actually require specific mechanisms to ensure efficient rRNA transcript elongation. Clearly, further *in vivo* studies of rRNA expression must be undertaken to address the question of the presence or absence of antitermination functions in *Bacillus*.

Lack of growth rate dependent regulation of promoter-*xylE* fusions.

The results presented in Chapter 1 demonstrated that *B. subtilis* and *E. coli* rRNA promoters were regulated in a growth rate dependent manner when fused to a chloramphenicol acetyltransferase gene and expressed in *E. coli*. Unexpectedly, it was found that this growth rate dependent expression could not be duplicated when these same promoters were fused to the *xylE* gene. It must be noted however, that the pAS-3 vector used for most experiments here differed from pKK232-8 used in Chapter 1 in that pAS-3 carried an internal transcription terminator which reduced expression of *xylE* by an average of 95% (Table 5). It was originally assumed that the efficiency of transcription
termination at tR1 varied as a function of growth rate and thereby masked any growth rate dependent expression which might be occurring. Others have noted that the number of transcripts which read-through a given termination signal could vary according to the strength of the promoters which initiate transcription (148). While the effects of growth rate on transcription termination have not been directly examined here, it is not unreasonable to assume that as the relative strength of a growth rate regulated promoter increased at higher growth rates, so too did the degree of transcription termination at tR1, thus effectively nullifying any growth rate dependent changes.

Growth rate dependent termination at tR1 if indeed it occurred, could not be the only explanation for the lack of an observable growth rate dependent response, as evidenced by the results obtained for the pTLXT-220B fusion plasmid (Fig. 22). This plasmid lacked the internal tR1 terminator of pAS-3 and carried the B. subtilis P2 rRNA promoter which previously had been shown to be growth rate regulated when fused to the CAT gene. If the lack of growth rate regulation of xylE-based fusion plasmids was due only to the presence of the tR1 terminator, then this fusion should have shown a normal growth rate dependent response. As can be seen (Fig. 22), pKK-220B also failed to produce an increase in catechol 2,3-dioxygenase activity with increasing growth rate. The reason for this is presently not
clear. Plasmid copy number determinations for the xylE vectors (data not shown) failed to show any significant difference between high and low growth rate conditions, as shown previously for the pKK232-8 vectors. It was observed however, that the functional half-life of xylE mRNA was significantly reduced in faster growing cells (G.B. Spiegelman, personal communication). Whether this could account for the lack of an apparent growth rate dependent response remains to be determined. The precise reasons are essentially irrelevant to the present investigations however, since from the data presented above it is clear that the xylE vectors have serious limitations if used to study the regulatory properties of cloned promoters as a function of changing parameters such as growth rate. Nevertheless, both pAS-3 and pTLXT-11 may still be useful as a means of quantitatively assessing the strength of cloned promoters as long as other environmental conditions are equivalent in all cases. The data in Table 5 for example, confirmed the previous observations (Chapter 1) that the B. subtilis rrnB P2 promoter was much more transcriptionally active than the upstream P1 promoter. For the promoters cloned in pAS-3, P2 was roughly 4 times more active than P1 at a cellular growth rate of 1.0 doubling/hr.; for the promoters in pTLXT-11, P2 predominated over P1 by a factor of 2. This variation in the ratio of P2 to P1 activity according to whether the cloning vector was pAS-3 or pTLXT-11 could be due to differential termination effects dependent on the absolute strengths of the cloned promoters as discussed above.
In conclusion, two general purpose promoter-probe vectors based on the *Pseudomonas putida* *xylE* gene have been developed here. Both are presently useable only in *E. coli* but have the potential for use (with modifications) in *B. subtilis*. Selection of recombinant clones carrying promoter fragments is simply and conveniently done by utilizing a color-change reaction. Promoters of average strength may be cloned into pTLXT-11 but very strong promoters require the presence of an internal terminator fragment so as to reduce the amount of *xylE* gene product synthesized. Thus, it has been suggested (although not rigorously proven) that overproduction of catechol 2,3 dioxygenase may be toxic to *E. coli* cells, an effect not previously noted for this product (126). In this regard, the pAS-3 vector has a significant advantage over other *xylE*-based vectors (126) in that very strong promoters can be maintained without detectable instability.

Vectors using *xylE* as an indicator gene have a serious drawback however, in that they cannot be used to study the regulatory properties of cloned promoters because of artifacts arising from the fusion system itself which may, under some conditions, obscure certain regulatory phenomena. It should be noted that this drawback may also extend to other *xylE*-based fusion systems (126, 149) and the appropriate controls must be
therefore considered. In spite of these difficulties, these vectors have been used to clone the promoter regions of the \textit{B. subtilis} and \textit{E. coli} rrnB operons in a manner analogous to that described in Chapter 1. While studies pertaining to the growth rate regulation of these promoters could not be done, it was nevertheless possible to define certain other features of the \textit{Bacillus} promoters which differed from the analogous \textit{E. coli} promoters. Specifically, by using the pAS-3 vector, it was concluded that the \textit{B. subtilis} rRNA promoter either lacked the antitermination functions found associated with \textit{E. coli} rRNA promoters, or if present, they were sufficiently different from those of \textit{E. coli} so as to be non-functional on \textit{E. coli} rho-dependent terminators. In order to gain more insight into these and other questions, parallel studies of rRNA promoter-fusion systems in \textit{Bacillus} must be undertaken.
Chapter 3

Attempts to construct bifunctional operon fusion vectors.

Introduction

As stated in the introduction to Chapter 2, the preferred next stage in this work would involve the transfer of an \textit{E. coli} promoter-fusion system into \textit{B. subtilis} in order that parallel \textit{in vivo} experiments could be performed. Ideally, the promoter expression vector used in the \textit{E. coli} experiments would be directly transferred to \textit{Bacillus}, so as to eliminate any potential problems which could arise from the use of two different expression systems in the two hosts. As detailed in Chapter 2, an initial step toward this goal was the construction of a promoter-fusion vector based on the \textit{Pseudomonas putida xylE} gene since this gene can be efficiently transcribed and translated in both \textit{E. coli} and \textit{B. subtilis} (126). An alternative approach, although technically more difficult, would have been to alter the ribosome binding site of the CAT gene on the pKK232-8 vector so that chloramphenicol acetyltransferase could be synthesized in \textit{B. subtilis}. It was shown however, that in \textit{E. coli} the level of \textit{xylE} gene expression was not strictly dependent on the transcriptional activity of the cloned promoter alone. Instead, the level of catechol 2,3-dioxygenase activity was influenced by other, poorly characterized, parameters thus
making the *xylE* vectors unsuitable for regulatory studies in *E. coli*. However, it was unclear whether similar problems with a *xylE*-based system would occur if this gene was expressed in *Bacillus*. It was decided to utilize the *xylE* vectors pTLXT-11 and pAS-3 in an attempt to construct promoter-probe plasmids which would replicate and express catechol 2,3-dioxygenase activity in both *E. coli* and *B. subtilis*. If feasible, this should allow an assessment to be made of the general utility of *xylE* vectors in the study of cloned promoters introduced into *Bacillus*. In addition, such vectors might be useful as the starting point for other bi-functional plasmids in which the *xylE* gene was replaced by another marker gene, one which would not present the problems associated with the expression of *xylE* in *E. coli*.

While there are no naturally occurring plasmids capable of replication in both *E. coli* and *B. subtilis*, several workers have created such bi-functional plasmids by the *in vitro* ligation of replicons native to each organism. One of the first such vectors was pHV14 (150) in which the *E. coli* plasmid pBR322 was ligated to pC194, a chloramphenicol-resistance plasmid originally isolated from *Staphlococcus aureus*. Transformation of pHV14 into *E. coli* resulted in the expression of ampicillin, tetracycline (from pBR322) and chloramphenicol (from pC194) resistance whereas only chloramphenicol resistance was expressed in *B. subtilis*. Since then, a large number of other bi-functional plasmids have
been created, most based on pBR322 as the E. coli replicon plus Gram-positive replicons such as pBD9, pBD64, or pUB110 (151). Frequently however, stability problems have been encountered with these bi-functional plasmids especially after transformation into a B. subtilis host (152). Usually, this resulted in spontaneous deletion of certain regions of either the Bacillus or the E. coli component of the plasmid, but the end result was a smaller replicon which was considerably more stable than its parent. Very recently some workers have described the construction of composite vectors which incorporated only the ori region from Gram-negative and Gram-positive plasmids plus various individual drug resistance markers and internal termination sites to ensure stability (141). By eliminating extraneous sequences, the size of such bi-functional vectors was considerably reduced and the problem of instability in either host seems to have been overcome. This section describes a number of attempts at creating bi-functional expression vectors based on pTLXT-11/pAS-3 and various Bacillus replicons, as well as composite vectors which are integratable into the B. subtilis chromosome.

Results


i) The first attempt at construction of a bi-functional
vector was made using the *E. coli* plasmid pAS-3 and the *B. subtilis* plasmid pBD9 (151). pBD9 was chosen because it has been shown to carry two selectable markers (kanamycin and erythromycin resistance) rather than only one, a feature which could prove useful in the selection of recombinant clones and also in monitoring plasmid stability. Both pAS-3 and pBD9 were cut at their unique *PstI* sites, ligated, and used to transform *E. coli* HB101 to erythromycin and kanamycin resistance. Ligation in this fashion resulted in the insertional inactivation of the ampicillin resistance gene of pAS-3 such that the recombinant plasmids only expressed the pBD9 markers. One of these clones, pAS3B, was found by restriction analysis to have the structure expected of a cointegrate plasmid. Digestion of pBD9 with *HpaII* resulted in 6 fragments (151, Fig. 24), the largest of which (3650 bp) contained the *PstI* site used in constructing the cointegrate with pAS-3. Digestion of pAS3B with *HpaII* would be expected to result in the loss of the 3650 bp fragment and the appearance of two new fragments both containing sequences from pBD9 and pAS-3. As seen in Fig. 24, digestion of pAS3B with *HpaII* resulted in the appearance of two new fragments of 1010 and 2660 bp, indicating that this plasmid was a true cointegrate between pAS-3 and pBD9.

Transformation of *B. subtilis* 168 with pAS3B resulted in the appearance of erythromycin-kanamycin resistant colonies at frequencies shown in Table 6. Since it was now established that
Table 6. Frequency of transformation with bi-functional vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>E. coli</th>
<th>B. subtilis</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAS3B</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>pCmTv-2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>pASTV-1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>pAS3C-E</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>pASUB-1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>pCm-2/pAS3C</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>pTV8</td>
<td>0</td>
<td>3</td>
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</tbody>
</table>
Figure 24. Restriction endonuclease digestion pattern of pAS3B.

Purified DNA was digested and electrophoresed through a 4% polyacrylamide gel as described in the Materials and Methods. Lane 1, φ29 digested with HindIII as molecular weight markers. Size of fragments corresponds to that given in Fig.1B and Fig.4. Lane 2, pAS-3 digested with HpaII; Lane 3, pAS3B digested with HpaII; Lane 4, pBD9 digested with HpaII. Arrows denote the two new fragments of 2660 and 1010 bp in pAS3B created from the 3650 bp (uppermost) fragment of pBD9 (see text for details).
pAS3B could replicate in *B. subtilis* and *E. coli*, the 427 bp *B. subtilis* *rrnB* promoter fragment was isolated from pKK-427B (Fig. 3A, Chapter 1), cloned into the SmaI site of pAS3B (analogous to the Sma site of pAS-3, Fig. 21) and used to transform *E. coli* HB101 to erythromycin, kanamycin resistance. Yellow colonies (+xylE) were selected at frequencies of about 1 in 20 after spraying recombinant colonies with a catechol solution. Of 10 +xylE clones analyzed, all carried plasmids but all plasmids were substantially smaller (by about 1000-1500 bp) than the original pAS3B vector (data not shown). Because of the large size of the recombinant plasmids (approximately 14 Kb) it was difficult to assess whether all were deleted to the same extent or whether there was some heterogeneity. Restriction endonuclease mapping was not performed. However, after digestion of the +xylE recombinants with EcoRI and BamHI, a fragment the same size as the authentic 427 bp promoter fragment could be liberated, indicating that the cloned promoter was probably unaltered. When + plasmid DNA from these +xylE recombinant clones was added to competent *B. subtilis* 168 cells, no erythromycin or kanamycin resistant transformants could be isolated. The deletion that occurred during the cloning of the 427 bp promoter therefore apparently encompassed some regions of the pBD9 portion of pAS3B that were necessary for replication in *B. subtilis*.

ii) In an attempt to reduce the size of the bifunctional
vector, a cointegrate between pAS-3 and pUB110 was constructed. This 4.5 Kb plasmid was originally isolated from *Staphylococcus aureus* (114, 151) but could replicate in *B. subtilis* and express kanamycin resistance in this host. Both pAS-3 and pUB110 were linearized with *EcoR1*, ligated, and used to transform *E. coli* HB101. Ampicillin-kanamycin resistant colonies which arose were all seen to carry plasmids larger than either of the original parental plasmids. The cloning strategy used here resulted in the loss of the upstream transcription terminator present in pAS-3; this apparently did not lead to read through transcription of the *xylE* structural gene since all of the ampicillin-kanamycin resistant colonies displayed a *xylE* phenotype. One of these plasmids, pASUB-1, was digested with *EcoR1* and found to give two fragments on agarose gels identical in size to *EcoR1*-linearized pAS-3 and *EcoR1*-linearized pUB110 (data not shown). This indicated that pASUB-1 was indeed a cointegrate between the two parental plasmids. *B. subtilis* 168 transformed with pASUB-1 DNA gave rise to kanamycin resistant colonies with the frequency shown in Table 6, but none of these were resistant to ampicillin as well, suggesting that the *bla* gene of pAS-3 was not expressed in *B. subtilis*.

The 427 bp *B. subtilis rRNA* promoter fragment was cloned into the *SmaI* site of pASUB-1 and *xylE* *E. coli* transformants isolated. These recombinants all carried plasmids of the expected size and all had promoter inserts of the correct size.
One of these plasmids, pASUB-427B, was used to transform \textit{B. subtilis} to kanamycin resistance. However, none of the colonies which arose displayed the \textit{xylE} phenotype. Analysis of the plasmid DNA from 5 of these kanamycin resistant \textit{B. subtilis} clones revealed in all cases, the presence of plasmids which were between 2 and 2.4 kb smaller than the original pASUB-427B. In this case therefore, the bi-functional pASUB-427B vector appeared to be stable in \textit{E. coli} but transfer to \textit{B. subtilis} resulted in deletion of certain pAS-3 related sequences involving the cloned promoter insert, the \textit{xylE} structural gene, or both.

iii) A number of bi-functional vectors based on pTLXT-11 or pAS-3 and fragments derived from the \textit{B. subtilis} plasmid pTV8 were constructed (153). This latter plasmid carried the temperature sensitive replication functions from pE194 as well as the complete Tn917 transposon (a macrolide-lincosamide resistance transposon from \textit{Streptococcus faecalis}). Transformation of pTV8 into a \textit{B. subtilis} host carrying a chromosomal copy of Tn917, followed by a temperature upshift to 42 C would result in the integration of the entire pTV8 vector into the chromosome by recombination in the region of Tn917 homology (153). It was anticipated that bi-functional vectors could be created which would replicate autonomously in \textit{E. coli} but could be stabilized in \textit{B. subtilis} by integration as a single copy into the chromosome. Figure 25 shows the structure of one set of these.
pCmTv-2 was created from pTLXT-11 (see Fig. 21) by insertion of a chloramphenicol acetyltransferase gene from pC194 into the PstI site of pTLXT-11, followed by insertion of a 6.8 Kb PstI - KpnI fragment from pTV-8 into the PvuI site of pCmTv-2 (see text for details). As indicated, this fragment contains roughly two-thirds of the Tn917 transposon as well as the temperature-sensitive origin of replication from pE194 (denoted as "ori"). All other sequences are as shown in Fig. 21. pASTV-1 is identical to pCmTv-2 but with the addition of the lambda trl transcription terminator into the HpaI site as seen in Fig. 21.

Restriction sites are: Ps, PstI; Pv, PvuI; E, EcoRl; S, SmaI; B, Bam HI; H, HpaI; K, KpnI; Sa, SalI; A, AvaI.
plasmids, pCmTv-2 and pASTV-1. Initially, the *bla* gene of pTLXT-11/pAS-3 was inactivated by inserting into the unique *Pst*I site a 1032 bp *Msp*I-*Mbo*I fragment carrying the chloramphenicol acetyltransferase gene of pC194 (146). The resulting plasmids, pCm-2/pAS3C, could still only replicate in *E. coli* but could potentially express chloramphenicol resistance in both *E. coli* and *B. subtilis*. A 6.8 Kb *Pst*I-*Kpn*I fragment from pTV8 (153) carrying the origin of replication and about two-thirds of the Tn917 transposon, was then inserted into the *Pvu*I site of pCm-2/pAS3C. The resulting vectors, pCmTv-2/pASTV-1, were both capable of replication in *E. coli* and in *B. subtilis* (see Table 6).

In addition, a smaller fragment from pTV8 was also inserted into pAS3C. A 3400 bp *EcoR*I fragment from pASTV-1 (see Fig. 25) was cloned into the *Pst*I site of pAS3C. This fragment contained only the pE194-derived origin of replication from pTV8 such that the new recombinant plasmid, pAS3C-E was able to replicate autonomously in *B. subtilis* (Table 6) but should not integrate into the chromosome since the Tn917 region of homology was lacking.
While pCmTv-2, pASTV-1, and pAS3C-E all appeared stable in *E. coli* and could replicate in *B. subtilis*, problems arose when attempts were made to insert the 427 bp *rrnB* promoter so as to activate the *xylE* gene. In all cases, *xylE* recombinants could be isolated but these either: a) showed no evidence of an intact *rrnB* promoter insert (while retaining the *xylE* phenotype in *E. coli*) and could be used to transform *B. subtilis* where they were phenotypically *xylE* or b) contained an intact 427 bp promoter but grew considerably slower than cells containing only the parental vector. In addition, these latter recombinant plasmids were considerably smaller than the original vector and were unable to transform *B. subtilis*.

Thus, as seen for previous bi-functional vectors, these plasmids also displayed serious stability problems coincident with attempts to insert the *B. subtilis* *rrnB* promoter.


Since attempts at constructing dual-origin bi-functional vectors had consistently failed, a different approach was taken in an attempt to obtain expression of the *rrnB-xylE* fusion in *Bacillus*. Other workers have shown that plasmids which are incapable of replication in *B. subtilis* may nevertheless become established in this host by integration into the chromosome if they carry a segment of DNA having homology to *B. subtilis*
chromosomal sequences (96, 154). The plasmid pAS3C (above) replicated only in *E. coli* but carried an antibiotic-resistance marker expressible in both *E. coli* and *B. subtilis*. Total chromosomal DNA was isolated from *B. subtilis* 168 and subjected to partial digestion with *PstI*. These fragments were then ligated to *PstI*-linearized pAS3C and used to transform *E. coli* HB101 to chloramphenicol resistance. Two clones containing plasmid DNA larger than the parental vector were identified and analyzed by digestion with *PstI*. As seen in Fig. 26, both recombinant plasmids contained inserts which could be liberated by *PstI*; pAS3C-168-4 contained one fragment of approximately 1800 bp whereas pAS3C-168-3 contained 2 fragments of about 1100 bp and 1600 bp. *B. subtilis* 168 could be transformed to chloramphenicol-resistance with either -168-3 or -168-4 at frequencies of 420 and 155 colonies per ug of plasmid DNA respectively. Plasmid DNA could not be detected in the transformants so it was assumed that chromosomal integration had occurred. To see if these vectors could express *xylE* activity after integration, the 427 bp *rrnB* promoter was cloned into the *SmaI* site of pAS3C-168-3 and *xylE* recombinants isolated from *E. coli* HB101. These *xylE* clones were stable in *E. coli* and carried a promoter insert of the correct size. When used to transform *B. subtilis*, chloramphenicol-resistant clones could be isolated at frequencies similar to that seen for the parental vector, but the *xylE* phenotype was only barely detectable even after the plates had
Figure 26. Restriction endonuclease digestion pattern of pAS3C-168 integrative plasmids.

Plasmid DNA was digested and electrophoresed through a 4% poly-acrylamide gel. Lane 1, pAS3C-168-4 digested with PstI; Lane 2, pAS3C-168-3 digested with PstI; Lane 3, Ø29 digested with HindIII as molecular weight markers. Relevant sizes are noted at the right, in base pairs.
been left for several hours after spraying with the catechol substrate. Catechol 2,3-dioxygenase activity from these clones could not be accurately measured using the standard assay conditions described in the Materials and Methods (activity less than 50 units). Thus it appeared that integrative vectors of this type were not suitable for the purposes originally outlined.

**Discussion**

The goal of the above experiments was to develop a means of transferring a functional operon fusion system from *E. coli* to *B. subtilis*. In this way, the regulation of cloned promoters could be studied in parallel in these two hosts. To this end, two approaches have been taken. Firstly, a set of dual-origin bi-functional plasmid vectors which could be used to shuttle a promoter-*xylE* fusion between *E. coli* and *B. subtilis* has been constructed; and secondly, an *E. coli* vector was modified such that it could integrate as a single copy into the *B. subtilis* chromosome. Unfortunately, neither of these approaches was entirely successful. In the case of the bi-functional vectors, the major problem appeared to be the stability of the chimeric plasmids, either in *E. coli* or when transferred to *B. subtilis*, or after insertion of promoter fragments. The bi-functional cointegrate plasmids pAS3B and pASUB-1 were apparently stable in *E. coli* and could transform *B. subtilis*, although at low frequencies. The observed differences in transformation
frequency from one plasmid to another may not be a reflection of the composition of the plasmids per se, but probably are due to differences in the degree of multimeric formations from one plasmid preparation to another. Canosi et al. (155) have shown that efficient transformation of competent B. subtilis cells was only achieved using multimeric forms of plasmid DNA. Plasmid multimers are generated in E. coli but this could vary for different plasmids and thus lead to differences in transformation efficiency.

Both pAS3B and pASUB-1 were rendered unstable by insertion of the 427 bp B. subtilis rrnB promoter and activation of the xylE gene. In the case of pAS3B, this resulted in extensive deletion of vector sequences such that recombinant plasmids could still replicate in E. coli but could no longer transform B. subtilis. Thus it appeared that the Bacillus-specific sequences on the chimeric vectors were targeted for deletion. In pASUB-1, a promoter-containing clone was stable in E. coli and could transform B. subtilis but the resulting B. subtilis recombinant plasmids were deleted and did not maintain a xylE phenotype. In this case therefore, an E. coli-specific sequence may be deleted although this was not directly verified.

A similar situation occurred with the pASTV/CmTv set of vectors in that they were stable in E. coli and could transform
B. subtilis. However, when the \textit{rrnB} promoter was inserted, deletions were generated either directly after transformation of \textit{E. coli} or after re-transfer to a \textit{B. subtilis} host. In no case could a stable \textit{xylE} phenotype be maintained in \textit{B. subtilis} using these autonomously replicating vectors. Instances of plasmid instability similar to these have been well documented and are a major problem in the development of an efficient cloning system for \textit{B. subtilis}. Grandi \textit{et al.} (156) for example, constructed a hybrid between the Bacillus plasmid pSA2100 and a pBR322 derivative carrying the \textit{E. coli} \textit{hisG} gene. Such hybrids were stable in \textit{E. coli} but suffered deletions to various extents when introduced into \textit{B. subtilis}. Similarly, Ostroff and Pene (152, 157) noted that \textit{B. subtilis} DNA sequences cloned in \textit{E. coli} and subsequently re-introduced into \textit{B. subtilis} via a bi-functional vector underwent severe deletions that involved both vector and insert sequences and were not prevented by the use of an \textit{r}_{\text{recE}} Bacillus host. The vector itself, without cloned inserts, was uniformly stable in both hosts. In addition, the problem of structural instability appeared to be due solely to the passage of cloned inserts through \textit{E. coli} prior to re-introduction into \textit{B. subtilis}.

The reason for the stability problems observed by these and other workers, as well as in this report, are not entirely clear. Ehrlich \textit{et al.} (150) have postulated the existence in Bacillus of a highly efficient recombination mechanism which is active on
sequences with only limited amounts of homology. Recombination between sequences within a cloned insert or between insert and vector sequences would lead to the formation of deleted variants which would soon predominate in the cell population. Supporting this hypothesis is the observation that *B. subtilis* plasmids recombine very frequently, possibly because they can exist as single-stranded DNA molecules within the cell (159). Alternatively, Ostroff and Pene (157) have suggested that some deletions may occur during the DNA processing events necessary for the uptake of plasmid DNA during transformation. Since the active transforming DNA molecules are chimeric trimers which are linearized upon contact with competent cells and subsequently recircularized after uptake (158), the large size of most bi-functional vectors could exceed the length of processed DNA and thus necessitate some deletion events (157). It may be of significance then that the hybrid vectors used here were relatively large, ranging from 11 to 15 Kb. Finally, Ostroff and Pene (157) have suggested that some as yet unidentified restriction-modification system of *B. subtilis*, different from the weak hsdM,R system thus far identified, could also generate instability in chimeric sequences modified by passage through *E. coli*. It must be noted however, that stability problems were not apparent when plasmids capable of integration into the *B. subtilis* chromosome were used as expression vectors (see below). It is possible that the multicopy nature of the bi-functional
hybrid vectors used above is a contributing factor to their instability in *B. subtilis*. It would seem therefore that the hypothesis put forth by Ehrlich *et al.* (150, see above) may be the most reasonable. Whatever the reason for the structural instability of the vectors used here, it is clear that bi-functional or hybrid fusion vectors of this type are unsuitable for the purposes intended, namely as a means of undertaking parallel studies of a particular gene sequence in two different host organisms.

A different approach to this goal was taken in the construction of the integratable expression vectors pAS3C-168-3 and pAS3C-168-4. Here it was reasoned that the introduction of a suitably large region of *B. subtilis* chromosomal DNA onto a plasmid vector which could not replicate in *B. subtilis* would lead to integration of the entire plasmid into the *Bacillus* chromosome via a homologous recombination event. This approach had previously been shown to be useful in the genetic mapping of a number of *B. subtilis* genes (154), including ribosomal RNA genes (96). Both pAS3C-168-3 and -168-4 were shown to contain *Bacillus* chromosomal sequences and both could transform *B. subtilis* 168 to chloramphenicol resistance while apparently not remaining as autonomously replicating molecules in the transformed cells. It must be pointed out however that proof of chromosomal integration, via Southern hybridization analysis for example, has not been demonstrated here, although the
circumstantial evidence as discussed above points to this being the case.

Even this however was not a completely suitable fusion system because although the xylE phenotype could be expressed in B. subtilis, the level of catechol 2,3-dioxygenase activity was too low to be accurately measured. Since the xylE mRNA was efficiently translated in Bacillus (126), the low level of expression was presumably due to a combination of two other factors. Because chromosomal integration had occurred, the copy number of the rrnB promoter-xylE fusion had been reduced to one per chromosome in B. subtilis in contrast to the multicopy, autonomously replicating state in E. coli. Overall xylE activity may therefore be expected to be reduced as much as 15-20 fold in Bacillus, based on the known copy number of pBR322 in E. coli (160). Additionally, the pAS3C-168 vectors contained the lambda tr1 transcription terminator proximal to the xylE gene. This terminator has recently been shown to be recognized by a B. subtilis sigma-55 RNA polymerase complex in an in vitro transcription system (141), so it can be assumed that it is operable to the same extent in vivo as well. This would further reduce the number of xylE-specific transcripts and reduce the overall catechol 2,3-dioxygenase activity to levels unmeasurable by the assay employed here. While it would be possible to remove the lambda terminator from pAS3C-168, this would have a serious
drawback in that the tandem \textit{rrnB} promoter-\textit{xylE} fusions would once again be unstable in \textit{E. coli} and make subsequent manipulation of the plasmids much more difficult.

In conclusion, a means of stably introducing strong cloned promoters into both \textit{E. coli} and \textit{B. subtilis} (via pAS3C-168-3) has been developed, but this approach did not allow measurements of the degree of expression of these cloned promoters. Because of this, it has not been possible to effectively compare the expression of the \textit{B. subtilis} \textit{rrnB} promoters in both \textit{E. coli} and \textit{B. subtilis}, as was the original intent. The major problem encountered here appeared to involve the introduction of promoter fusion vectors into \textit{B. subtilis}. Other mechanisms, possibly the transfer of only the promoter-\textit{xylE} gene as a portable "cassette" onto a Bacillus phage, may have to be considered.

\textbf{Summary and Concluding Remarks}

The three chapters presented above outline a means whereby the promoter region of the \textit{B. subtilis} \textit{rrnB} ribosomal RNA operon can be cloned and expressed in \textit{E. coli}. This has been achieved through a fusion of the promoter to an assayable marker gene but of two such fusion systems tested, only one was found to be a valid means of assessing promoter activity \textit{in vivo}.

A number of operon fusion-type expression vectors were
created based on the catechol 2,3-dioxygenase (\textit{xylE}) gene of \textit{Pseudomonas putida}. While these vectors may be generally useful in the cloning and maintenance of both weak and very strong promoters, they were not suitable for assessing the transcriptional activity of cloned promoters when the host cells were placed under different physiological conditions. Under such conditions, it appeared that the \textit{xylE} operon fusion system was sensitive to other, ill-defined, parameters which masked or obscured the true transcriptional activity of the cloned promoters. When using the \textit{xylE} system, a comparison of the transcriptional activity of different promoters would therefore only be valid if the growth conditions of the host cells were equivalent in all cases.

Furthermore, attempts were made to modify the original \textit{xylE}-based fusion vectors such that they could serve as promoter-probe or expression vectors in both \textit{E. coli} and \textit{B. subtilis}. This was based on previous observations that, unlike most other Gram-negative genes, the \textit{xylE} messenger RNA could be efficiently translated in \textit{B. subtilis}. These attempts led to the construction of a number of bi-functional fusion vectors which were shown to replicate in both \textit{E. coli} and \textit{B. subtilis}. However, once promoters were inserted to activate transcription of the \textit{xylE} gene, serious stability problems arose which prevented the expression of any such promoter-fusion system in
B. subtilis. These stability problems could apparently be avoided if the operon fusion was integrated as a single copy into the B. subtilis chromosome. Unfortunately, expression of the xylE phenotype was very poor under such conditions because of the reduced copy number and because of the necessity of retaining a transcription terminator between the cloned promoter and the xylE structural gene. While these vectors would undoubtedly be useful for other purposes (i.e. cloning and expression of very strong homologous or heterologous promoters in E. coli; integration of cloned sequences into the B. subtilis chromosome), they did not fulfill the original intent of providing a means of studying the in vivo regulation of a given promoter in two different hosts. Although it was only possible to study the expression of the B. subtilis rrnB promoters in a heterologous E. coli background, these studies nevertheless provided some substantial insight into the regulation of these promoters, and when compared to the native E. coli rRNA promoters, suggested that present models to account for the growth rate dependent regulation of rRNA synthesis may be overly simplistic.

In this system, the B. subtilis rrnB tandem promoters, or subclones thereof, were fused to the gene for chloramphenicol acetyltransferase (CAT) on a multi-copy plasmid that permitted the maintenance of strong promoters. The in vivo promoter activity in E. coli could be indirectly determined by measuring the CAT specific activity. A number of control experiment showed this
measurement to be entirely valid since the CAT specific activity was directly proportional to the amount of CAT gene messenger RNA produced by the promoter fusion. It was observed that the \textit{B. subtilis} rrnB promoters were expressed in a growth rate dependent manner in \textit{E. coli}, in the same fashion as the native \textit{E. coli} rrnB promoters.

While it had been known for some time that a number of \textit{B. subtilis} genes could be transcribed with reasonable efficiency in \textit{E. coli}, the findings presented here demonstrated that higher level regulatory mechanisms, at least as far as ribosomal RNA synthesis is concerned, may also be functionally interchangeable between these two evolutionary divergent organisms. Whether this conservation is true only for those factors regulating expression of rRNA genes remains to be determined, but the results presented here do suggest a strong evolutionary conservation among components of the transcription-translation apparatus of Gram-negative and Gram-positive organisms. It is important to point out however that the possible conservation of higher level regulatory mechanisms may not extend to all genes or operons shared by \textit{E. coli} and \textit{B. subtilis}. This is evidenced by the fact that very few \textit{E. coli} genes or promoters function efficiently in \textit{B. subtilis}, indicating that \textit{Bacillus} is less flexible in terms of the types of foreign sequences that it can regulate. Clearly, further \textit{in vivo} studies of \textit{B. subtilis} genes and promoters in
Bacillus are required.

Furthermore, it is clear from this study that significant differences do exist when the expression of *B. subtilis* and *E. coli* rRNA promoters are examined more closely. By separating and individually cloning the upstream P1 and downstream P2 *B. subtilis* promoters, it was shown that the P2 promoter was the more active, growth rate regulated promoter of the tandem pair. Conversely, the *E. coli* rrnB promoters showed the reverse pattern of expression in that P1 was the strong, regulated promoter. Additionally, others have found that deletion of sequences upstream of the -35 site of the *E. coli* P1 promoter resulted in a significant reduction in the overall level of expression of the P1 promoter. Here however, it was shown that deletion of the analogous sequences from the *B. subtilis* rrnB promoter region had no effect on downstream expression. Furthermore, studies with the terminator-containing xylE fusion vector indicated that transcripts emanating from the *E. coli* rrnB promoters could efficiently read through a downstream rho-dependent terminator. Transcripts from the *B. subtilis* promoters were largely blocked by this terminator, indicating that antitermination functions were either not present in *B. subtilis* rRNA promoters or simply were not functional in a heterologous *E. coli* host.

It appears therefore that a precise analogy to *E. coli* rRNA
promoters is not necessary in order that a characteristic growth rate dependent response be elicited; certain features of these promoters, as illustrated by the \textit{B. subtilis} \textit{rrnB} promoters, can be altered without diminishing their response to increasing cellular growth rate. For example, the placement of a strong, regulated promoter upstream of the weaker, non-regulated promoter is unnecessary since this could be reversed in \textit{B. subtilis} without any loss of growth rate regulation. Secondly, the highly A-T rich sequence located upstream of the \textit{-35} region of all \textit{P1} promoters in both \textit{E. coli} and \textit{B. subtilis} is not required for either enhancement of the overall level of expression or in the expression of a growth rate dependent response since this region could be completely deleted from the \textit{B. subtilis} promoters without any change in the pattern of expression. In this regard it may be necessary to re-evaluate some features of the various mechanisms proposed to explain the control of growth rate dependent gene expression in \textit{E. coli}, as pointed out in the Discussion (Chapter 1).

From the evidence presented here it has been concluded that the mechanisms that ensure the growth rate dependent synthesis of rRNA do indeed act at the level of rRNA operon promoters but do so in ways that may be more subtle than previously anticipated. Possibly this could involve some sequence-specific conformational changes in the DNA surrounding growth rate regulated promoters, but changes that can be brought about by a number of different
but analogous sequences. In addition, there may be an interaction between the P1 and P2 promoters which serves to "fine-tune" the level of rRNA synthesis to the extent appropriate for a given set of growth conditions, as postulated in Chapter 1. Furthermore, the possible interactions between the individual rRNA operons on the chromosome, or whether they are all regulated to the same extent, are questions which remain unanswered. Clearly, further in vivo and in vitro studies are necessary to address these points but with the operon fusion vectors described and tested here, such studies may now be possible.
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