Ribosome synthesis during the cell cycle of *Rhodomicrobium vannielii*.

Christopher John Oakley,
B.Sc. (Hons) Warwick.

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University of Warwick, Department of Biological Sciences.

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I should like to thank my supervisor, Dr. Crawford Dow for his encouragement, guidance and carefree joviality.

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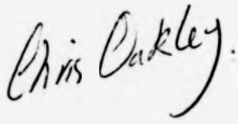
Declaration

I hereby declare that the work described in this thesis was conducted by myself, under the supervision of Dr. C. S. Dow.

None of the information contained herein has been used in any previous application for a degree.



Christopher J. Oakley

Summary.

A method for the purification of *R. vannielii* ribosomes was developed. The sizes of the two major rRNA's of *R. vannielli* were shown to be comparable to the 23S and 16S rRNA's of *E. coli* though the ribosomes themselves were smaller with subunit of sizes 46S and 26S.

^{32}P-orthophosphate pulse labelling showed that rRNA synthesis in *R. vannielii* occured at a constant rate throughout a four hour period of swarmer cell maturation and reproduction. However, due to the failure of dark inhibited swarmer cells (grown photoheterotrophically under anaerobic conditions) to incorporate any of the several nucleotide precursors tested, examination of the rate of rRNA synthesis for this cell could not be carried out by this approach. In order to circumvent possible variations in membrane permeability, a method was developed to isolate membrane associated nucleoids from *R. vannielii*. *In vitro* transcriptional run off from these nucleoids was achieved but did not prove to be reproducible.

Dark inhibited swarmer cells were shown to be competent in ^{35}S-methionine uptake. Pulse labelling of r-proteins with this radiolabelled amino acid showed that r-protein synthesis, though reduced in the inhibited swarmer cell in comparison with its differentiating counterpart, does occur. This result in a cell that exhibits no increase in biomass implies that ribosome turnover occurs.

Ribosomal RNA sequences were mapped on Southern blotted restriction digests of *R. vannielii* genomic DNA. The results indicate that this organism has at least two conventional eubacterial rRNA operons on its chromosome, though other atypical ribosomal sequences were found. The significance of these results is discussed.

Two *R. vannielii* rDNA clones were isolated and characterized: pC01 and pC02 are 8.8 and 9.1 kb respectively and each contains a single 16S rRNA coding sequence. The closest linkage that these 16S genes can have to another rRNA gene is 2 Kb. This result further suports the proposal that *R. vannielii* possesses atypical ribosomal sequences.

Three possible mechanisms by which ribosome synthesis may be influenced in the dark inhibited swarmer were investigated. No evidence was found for the action of a stringent response, DNA rearrangements or variations in DNA methylation.

Abbreviations

Ap	Ampicillin, sodium salt.
ATP	Adenosine triphosphate.
bp	Base pairs.
BSA	Bovine serum albumin.
Ci	Curie.
Cm	Chloramphenicol.
cpm	Counts per minute.
dATP	Deoxy-adenosine triphosphate.
dCTP	Deoxy- cytidine triphosphate.
dGTP	Deoxy-guanosine triphosphate.
DNase	Deoxyribonuclease.
DRS	Direct repeat sequences.
EDTA	Ethylenediaminetetra-acetic acid.
GDP	Guanosine diphosphate.
GTP	Guanosine triphosphate.
HPN	Highly phosphorylated nucleotide.
IRS	Inverted repeat sequences.
h	Hour.
kb	Kilobases.
MPa	Mega-Pascals.
Mr	Relative molecular mass.
OD	Optical density.
ppGpp	Guanosine tetraphosphate.
pppGpp	Guanosine pentaphosphate.
RNase	Ribonuclease.
RS	Repeated sequences.
S	Svedberg.
SDS	Sodium dodecyl sulphate.

Tc	Tetracycline.
TCA	Trichloroacetic acid.
TEMED	N,N,N,N' tetramethylenediamine.
Tm	Melting temperature.
Tris	Tris-hydroxymethylaminomethane.
TTP	Thymidine triphosphate.
5mC	5-methycytosine.
6mA	6-methyladenosine.

Dedication

To the five most important people in my life.

Chapter 1

Introduction

1.0. General Introduction.

Over the past few decades a great deal of research, time and effort has been invested in developing our understanding, at the molecular level, of how cells orchestrate their cellular processes during growth and differentiation. Although the ultimate aim must be to understand our own cellular functions, the prokaryotes have been allocated a large portion of this attention due largely to the relative ease with which they can be manipulated both biochemically and genetically. Research on prokaryotes has served both to pioneer an understanding that may be extrapolated to eukaryotes as well as to provide an interesting field of research in its own right. There are many important parallels to be drawn between the regulatory systems of prokaryotes and eukaryotes, though important differences must not be overlooked eg. tandem gene amplification as seen for the chorion protein genes of *Drosophila* (Spradling & Mahowald 1980).

Prokaryotic organisms exhibit a wealth of variety in their morphogenetic and differentiation events. Several organisms lend a number of advantageous properties to the study of these processes and have been selected for more detailed studies. The cell cycle has been most extensively studied in the monomorphic organism *E. coli*, yet even here little is understood of the fundamental control mechanisms. The simplicity of this organism's symmetric binary fission is however, far from typical in the prokaryotic world. The organisms *Caulobacter crescentus* (Ely & Shapiro 1984) and

Hyphomicrobium (Moore 1981) exhibit dimorphic vegetative cell cycles with distinct parent and progeny cells. *Geodermatophilus* (Ishiguro & Wolfe 1970) and *Arthrobacter* (Clark 1979) are polymorphic organisms in which changes in cellular morphology can be induced by changes in the nutrient conditions. Sporulation in *Bacillus subtilis* (Losick & Youngman 1984) is also well studied and represents a diversion from a wholely vegetative cell cycle and is induced by nutrient depletion. A higher order of organisation is found during both *Myxococcus* sporulation (Kaiser 1984) and heterocyst formation in *Anabaena* (Carr 1979), where intercellular communication occurs. Finally, the polymorphic organism *Rhodomicrobium vannielii* (Whittenbury & Dow 1977) which during its simplified cell cycle exhibits a similar distinction between parental and progeny cells to that seen in *C. crescentus* and *Hyphomicrobium*. Initiation of progeny swarmer cell maturation (an entirely vegetative process) is also under environmental control, in the form of light (when *R. vannielii* is grown photoheterotrophically under anaerobic conditions). In addition, it is possible that intercellular communication can occur between parental cells via their interconnecting prosthecae, though this question has never been addressed.

The aim of this thesis is to study the regulation of ribosome synthesis during *R. vannielii* swarmer cell maturation and reproduction. By far the majority of studies on the control of ribosome synthesis (and the stringent response, a known modulator of ribosomal synthesis) have

been carried out in *Escherichia coli*. For this reason I shall discuss these topics with respect to *E. coli*, having first described its own cell cycle. Two further mechanisms that are known to influence gene expression have been studied and will be discussed. These are, DNA rearrangement and site specific DNA methylation. Salient features will then be drawn from other selected prokaryotic differentiation systems followed by an introduction to the cell cycle of *R. vannielii* upon which this work is based.

1.1. The *Escherichia coli* Cell Cycle.

In many ways *E. coli* is the organism of choice in which to study prokaryotic development and the concomitant regulation of gene expression. It is easily manipulated, has arguably the simplest cell cycle and is the best understood organism both biochemically and genetically. However, this simplicity also has disadvantages ie. growth, septation and DNA replication are the only conspicuous points of reference within cell cycle.

E. coli is a simple rod-shaped cell that in growth, typically doubles its length prior to division by transverse fission into two cells of equal length. At the optimum growth temperature of 37^{o}C the minimum doubling time (td) is 20 minutes. As the growth rate increases both cell length and width increase such that the ratio between the two is constant thereby retaining the cells shape (Marr *et al* 1966). For any constant growth rate it has been shown that there is

little if any change in cell diameter. The minimal unit cell length (L_u) and the minimal unit cell volume (V_u) are observed in a new post divisional cell from a culture in which the growth rate tends towards zero (ie, the minimal unit cell).

The *E. coli* chromosome is a circular covalently closed molecule. Chromosome replication begins at a region designated *oriC* and proceeds at a constant rate, bi-directionally to completion in 40 minutes ie. the C period (Cooper & Helmstetter 1968). For td's below 100 minutes the C period is independent of growth rate (Donachie 1981). Completion of DNA replication marks the onset of septum formation, which takes 20 minutes to complete ie. the D period. A basic cell cycle would therefore be seen in a cell with a td of 60 minutes. In this situation one would observe a 40 minutes C period for DNA replication followed by a 20 minute D period for septation. However, this is rarely the case.

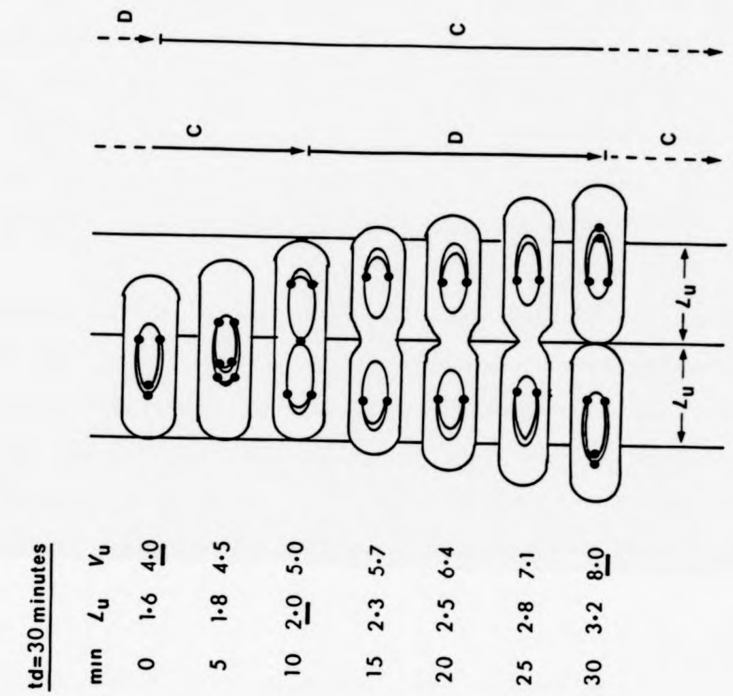
A td longer than 60 minutes is accommodated by the inclusion of a G1 period, prior to the C period, during which cell growth occurs but cell septation and DNA replication do not (figure 1.1.a.). Shorter td's are accommodated by overlapping C and D periods (figure 1.1.b.), with the periodicity at which DNA replication is initiated matching the 'ideal' td to ensure that each progeny receives a full chromosome. However, for all growth rates a number of basic rules apply. Chromosome replication is initiated when the cell volume reaches $2V_u$ and septation is initiated

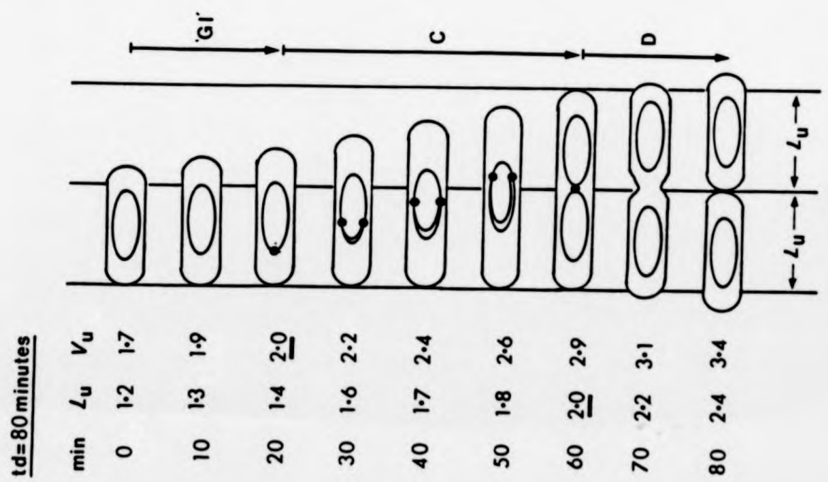
Figure 1.1.

The *E. coli* Cell Cycle.

The figure shows cell growth, DNA replication and cell division for an ideal *E. coli* cell cycle. Cell volumes and lengths are expressed as multiples of the minimum unit cell volume (V_u) and length (L_u) respectively. For all doubling times (td's) the time taken to carry out DNA replication (40 minute C period) and cell septation (20 minutes, D period) is invarient. DNA replication is initiated at $2V_u$ and cell division at $2L_u$. Doubling times longer than 60 minutes (C+D) are facilitated by the inclusion of a variable G1 period prior to C (Figure 1.1.a; td = 80 minutes) in which cell growth occurs, but DNA replication does not. Doubling times shorter than 60 minutes are facilitated by overlapping C and D periods (figure 1.1.b; td = 30 minutes).

Figure taken from Donachie (1981).





when the cell length reaches $2L_u$. Once the commitment to division has occurred at $2L_u$, a cell will go on to divide even if DNA and protein synthesis is halted. Cells that are less than $2L_u$ in length grow by elongation from one pole where as growth is initiated at the second pole once $2L_u$ is reached (Begg & Donachie 1977).

Initiation of septation is also dependent upon the completion of a round of chromosome replication with a subsequent initiation of chromosome replication setting up a block to any further initiation of septation. Cessation of this block requires the completion of a round of chromosome replication plus a further seven minute period of protein synthesis (Jones & Donachie 1973).

By delaying the completion of chromosome replication, it is possible to reduce the time interval between termination of DNA synthesis and cell separation from 20 minutes to a minimum of 7 minutes (Meacock & Pritchard 1975). This result suggests that not all the processes of septation are dependent on the completion of chromosome replication.

It is possible to block cell division such that cell growth continues to form long filaments. In these cases it is observed that at each doubling of L_u, one potential division site is formed. If the block is removed, septations are initiated at positions separated by 1 unit cell length (for cells at that growth rate). The number of potential division sites increases by the series 1, 3, 7 etc. (Donachie & Begg 1970).

1.1.1. Control of the *E. coli* Cell Cycle.

One may suspect that the regulation of the *E.coli* cell cycle would be conducted by specific activators or inhibitors and that these would be synthesized only at specific points in the cell cycle. An examination of the rates of synthesis of 750 polypeptides from various cell stages by 2D PAGE allowed no such variations to be resolved (Lutkenhaus *et al* 1979). Proposals were therefore been made that cell cycle events may be triggered by the cell physically reaching a particular size with respect to that of the minimal cell. Alternatively, it has been postulated that events are triggered by a critical concentration of a 'control molecule' that is sythesized at a constant rate but is accumulated during cell growth. However, it should be emphasized that the examination carried out by Lutkenhaus *et al* (1979) would fail to resolve low concentration regulatory polypeptides. To date some 50 genes have been implicated in having a direct effect upon morphogenesis in *E. coli* K-12 and these have been divided into nine functional groups (Donachie *et al* 1984). Hopefully, detailed characterization of these genes will provide an understanding of the fundamental mechanisms that regulate cell growth and division in this organism.

1.2. The *E. coli* Ribosome.

The ribosome is the site of protein synthesis in the cell. In *E. coli* this is a large molecular structure of

size 70S that can be further subdivided into two subunits, one small and one large, of 30S and 50S respectively. The whole 70S ribosome is constructed from 52 different ribosomal proteins (r-proteins) and 3 non-identical ribosomal RNAs (rRNA). The small subunit contains a total of 21 r-proteins and a 16S rRNA species (1541 nucleotides in length) while the large subunit has a total of 33 r-proteins and two different rRNA species of 23S and 5S (2904 nucleotides and 120 nucleotides respectively). These components are present in only one copy per ribosome with the exception of L7/L12 and L26/S20, where both pairs derive from the same gene ('L' denotes an origin in the large subunit and 'S', the small subunit).

The ribosomes may constitute as much as 40% of the dried mass of the cell and almost all the cells ribosomes will be actively engaged in protein synthesis. Free rRNA or r-proteins are only found in very small quantities and degradation of ribosomal components is insignificant, with the exception of that of rRNA during slow growth (Gausing 1977, Dennis 1974). There is, therefore, an obvious requirement to coordinate the synthesis of all 55 components to achieve an equimolar balance. In addition, ribosome synthesis represents a large energy drain on the cell and as such must be kept efficient. Under most growth conditions the cellular concentration of ribosomes is proportional to the growth rate of the cell, that is ribosome numbers rather than their activity are modulated to balance the protein synthesizing capacity with the growth rate.

1.2.1. Introduction to the Regulation of Ribosome Synthesis in *E. coli*.

The literature on ribosome biosynthesis is both vast and in many cases involved. The best way in which to introduce the topic is to give a narrow and undoubtedly misleading overview of the subject to act as a foundation on which to build.

'Free ribosomes', ie those excess to cellular requirements for a particular growth rate, mediate a feedback inhibition (possibly via guanosine tetraphosphate) to modulate rRNA transcription. The resulting decrease in the level of rRNA leaves specific repressor r-proteins (1 per operon that would normally bind to the rRNA) free to bind their own polycistronic r-protein mRNA and thereby inhibit its translation. In this way the rate of ribosome synthesis can be coupled to the growth rate of the cell (see figure 1.2).

An increase in nutrient availability results in the utilization of 'free ribosomes', thereby releasing the inhibition of rRNA transcription and subsequently r-protein translation. The result is an active synthesis of ribosomes until a small surplus is re-established.

1.2.2. Organisation of the r-Protein Genes in *E. coli*.

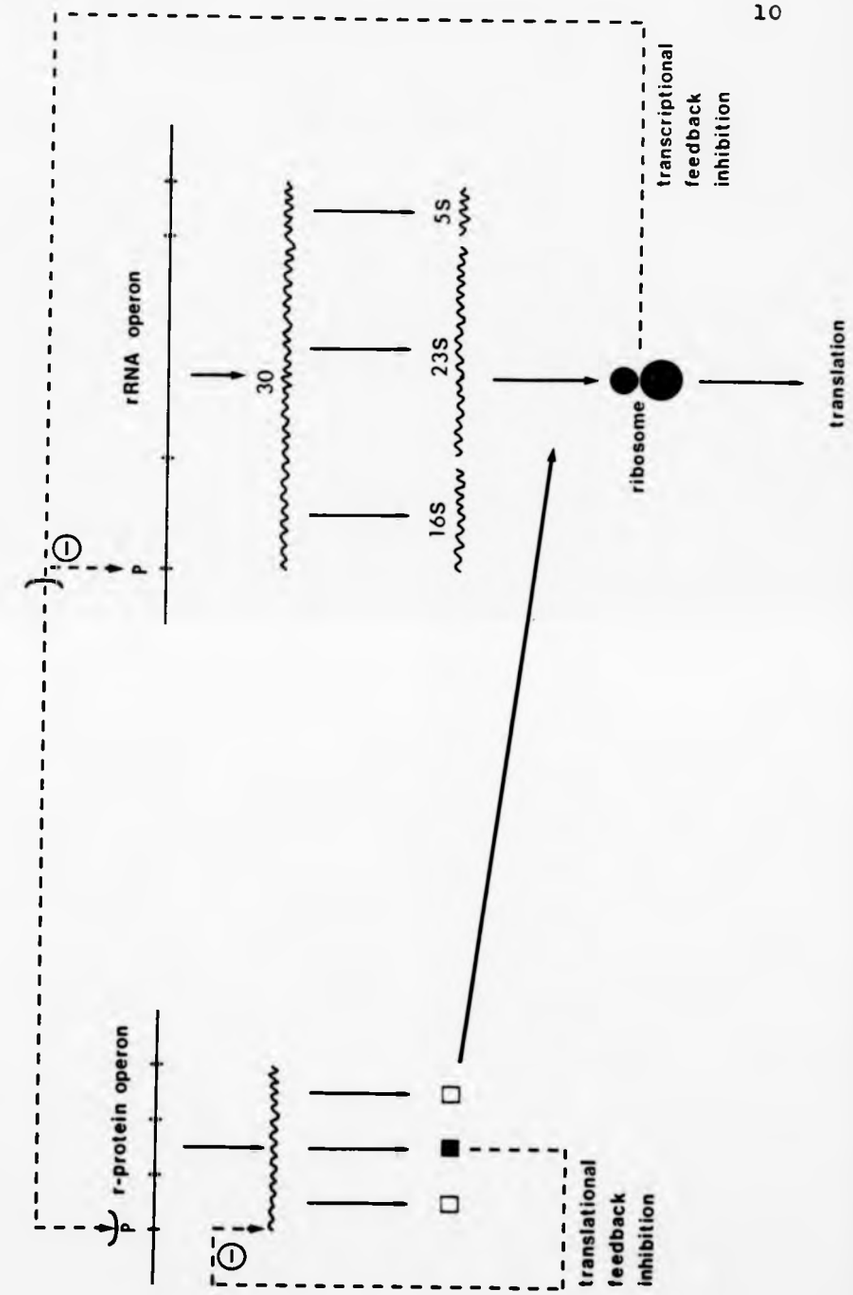
Figure 1.3 shows the location of the ribosomal protein genes/operons (represented by protein product) and rRNA

Figure 1.2.

Regulation of Ribosome Synthesis in *E. coli*.

The figure shows the synthesis of r-protein and rRNA from typical operons with their subsequent assembly into a 70S ribosome (RNA is depicted by wavey lines). Solid arrows indicate the normal process of ribosome synthesis.

Newly synthesized ribosomal proteins have a 'preference' for interacting with rRNA and hence ribosome assembly, but in the absence of rRNA, certain r-proteins can bind to their own mRNA and inhibit any further translation. Similarly, ribosomes show a 'preference' for active translation, a process that is unable to occur eg. in the absence of amino-acyl tRNA. If unused, 'free ribosomes' can mediate transcriptional feedback inhibition of the rRNA operons (and to an extent of the r-protein operons). In this way it can be seen that 'excess' ribosomes mediate the inhibition of rRNA transcription which in turn leads to the translational inhibition of r-protein synthesis.



operons (*rrn*) on the *E. coli* genetic map. It can be seen that the r-protein genes are scattered throughout the chromosome. Some r-protein genes are transcribed individually and some in operons containing up to 12 genes. The transcriptional organization of most of these operons is known and indicated. Interestingly, a number of non-ribosomal genes are found to be co-transcribed within these r-protein operons and these are identified in figure 1.3's legend.

1.2.3. Regulation of r-Protein Synthesis by Translational Feedback Inhibition in *E. coli*.

As stated above the main control of r-protein synthesis is via translational feedback and control. Figure 1.4 shows seven operons in which this effect has been characterized. In each case one r-protein, the translational repressor (identified by a box), can inhibit translation from the polycistronic mRNA derived from its own operon. Each of the repressor proteins are known to bind rRNA both strongly and specifically during the process of ribosome assembly. These proteins are able to recognize the same general structural features on their own mRNAs that they recognize on rRNA (Nomura et al 1984). The binding site for translational feedback regulation is situated at or near the translational initiation site of the first gene in the operon. Homologies in nucleotide sequence and/or proposed secondary structure between the rRNA binding sites and the binding sites for translational feedback regulation were recognized for r-proteins S4, S7, S8, L4, L1 and L10

Figure 1.3.

The Positions of the Ribosomal Genes on the Genetic Map of *E. coli*.

Ribosomal protein genes are represented by gene product and the seven rRNA operons are represented by their genetic nomenclature (*rrn*). Details of transcriptional organization are indicated by arrows. Several non-ribosomal genes are co-transcribed with the ribosomal genes and these include : RNA polymerase subunits α , σ , β and β'; elongation factors EF-Tu, EF-Ts and EF-G; DNA primase (*dnaG*); tRNA (m'G) methyl-transferase (*trmD*); a component of the secretion apperatus (*secY*) and a protein of unknown function X (Cierretti *et al* 1983).

The position of the origin of DNA replication *OriC* is also detailed.

Figure taken from Nomura *et al* (1984).

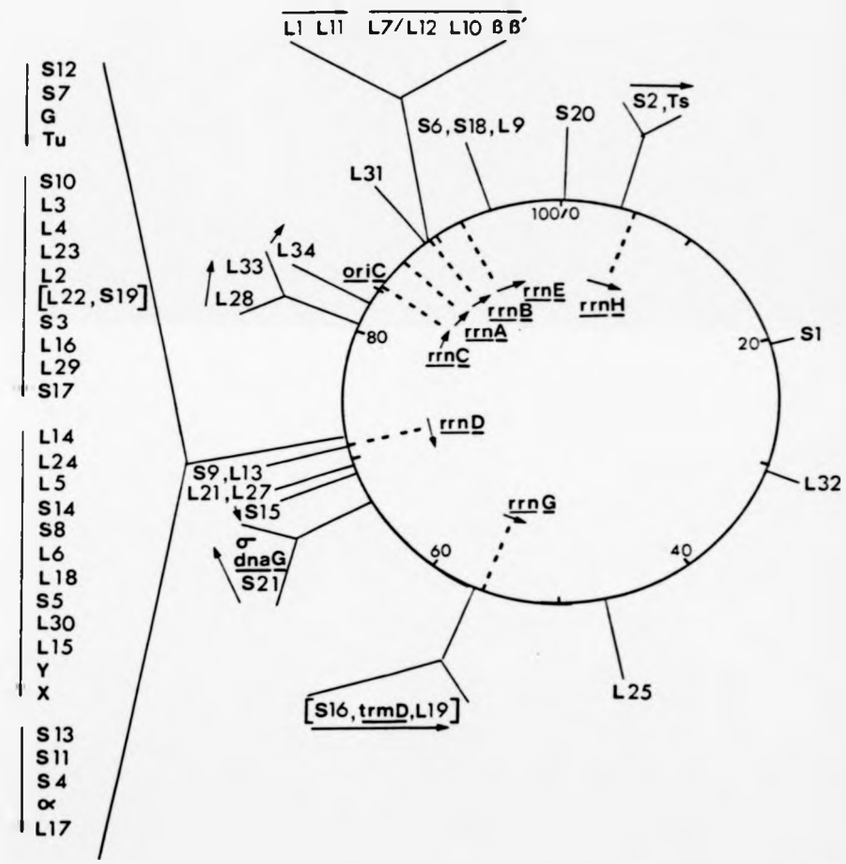
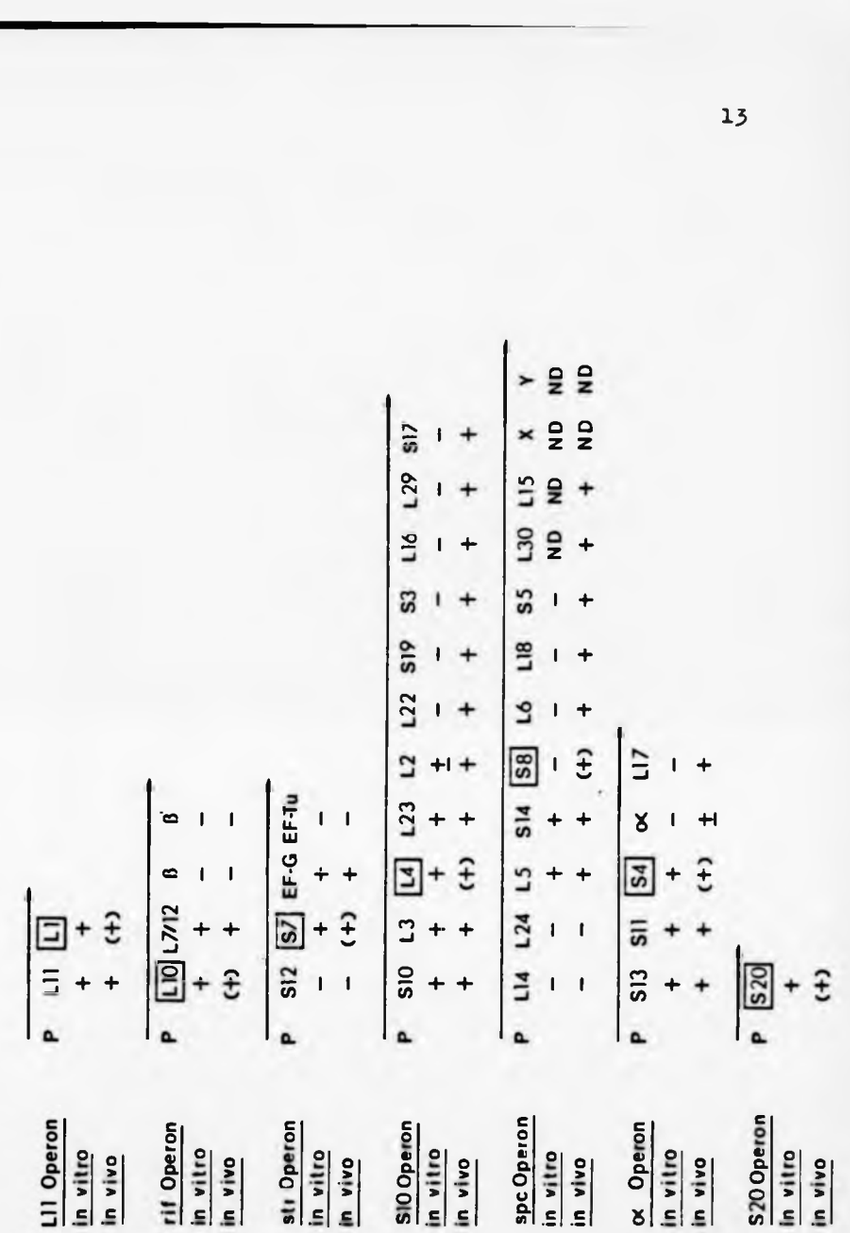


Figure 1.4.

Regulation of Gene Expression for the r-Protein Operons of *E. coli*.

The figure shows the organization of seven r-protein operons. For each, transcription runs from left to right being initiated at the promoter, P. One r-protein from each operon is able to repress translation from its own mRNA. This repressor r-protein is identified by a box and the effect of this repressor on each of the genes in its operon is indicated : + ,inhibition; −, no significant effect; +, weak inhibition; (+), inhibition presumed to occur *in vivo*; ND, not determined. Refer to figure 1.2 for the identity of non-ribosomal genes.

Figure taken from Nomura *et al* (1984).



(Yates & Nomura 1980, Johnson *et al* 1982, Olins & Nomura 1981, Gourse *et al* 1981, and Branlant *et al* 1981). This region includes the ribosome binding sites and translational initiation sites. L1 for example recognizes a stem and loop structure that contains at least 3 G-C base pairs for stem formation. Alteration of 2 of these base pairs relieves L1 feedback inhibition (Nomura *et al* 1984). Obviously, the binding to rRNA is favoured over mRNA binding. This ensures that ribosome assembly takes precedence over feedback inhibition, but in the absence of rRNA, translation of r-protein would be inhibited. It has been suggested that even those r-proteins that do not bind rRNA directly during assembly, could, by virtue of the specific r-protein to r-protein interaction, mediate an interaction with their mRNAs (Yates & Nomura 1981).

The effectiveness of this system can be demonstrated by gene dosage experiments. The number of copies of a r-protein operon can be elevated by introducing additional genes on plasmid vectors. This results in an increase in the rate of r-protein mRNA synthesis approximately in proportion with the gene dosage. However, the rate of r-protein synthesis is not significantly increased, indicating the action of a post-transcriptional regulatory mechanism. A further example of feedback translational regulation is documented for gene 32 of phage T4 (Lemaire *et al* 1978).

As translation of the downstream cistrons on the mRNA is usually dependent upon the translation of the first, the

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As translation of the downstream cistrons on the mRNA is usually dependent upon the translation of the first, the

cistrons are said to be translationally coupled; that is, independent translational initiation of distal cistrons is prevented. How this coupling functions is unclear. It has been proposed that the translational initiation sites for downstream cistrons are sequestered in RNA secondary and/or tertiary structures and that these interfere with ribosome access (Nomura *et al* 1984). The downstream cistrons may only become available during translation of the first cistron allowing either continued translation by the original ribosome or attachment of a second.

Precedents for translational coupling are found for the RNA phage R17 replicase gene (Gold *et al* 1981), the T4 rII system (Sarabhai & Brenner 1967) and the *lacI* gene system (Steege 1977). In the case of the *rif* operon, a new ribosome must be able to bind to the L10 - L7/L12 junction as L7/L12 is required in superstoichiometric amounts (Yates *et al* 1981). Interestingly, the L10-L7/12 inter-cistronic distance is 67 nucleotides, where in cases of equimolar synthesis this distance is relatively small; eg. the L11 - L1 distance is 3 nucleotides (Post *et al* 1979).

Consideration must also be given to the exceptions to this scheme. Studies have failed to demonstrate post--transcriptional control for the S21, L21 and L27 genes (Takata 1978). In these cases the regulatory mechanism is not known. Also, with the exception of EF-G, the synthesis of the non-ribosomal gene products, that are also encoded within the regulated r-protein operons appears to be

insensitive to feedback inhibition by the repressor r-proteins. The synthesis of β and β' appears to be modulated by a number of mechanisms, attenuation (Barry *et al* 1979), mRNA processing (Barry *et al* 1980) and feedback inhibition by RNA polymerase (Yura & Ishihama 1979, Scaife 1976).

1.2.4. Transcriptional Regulation of r-Protein Synthesis in *E. coli*.

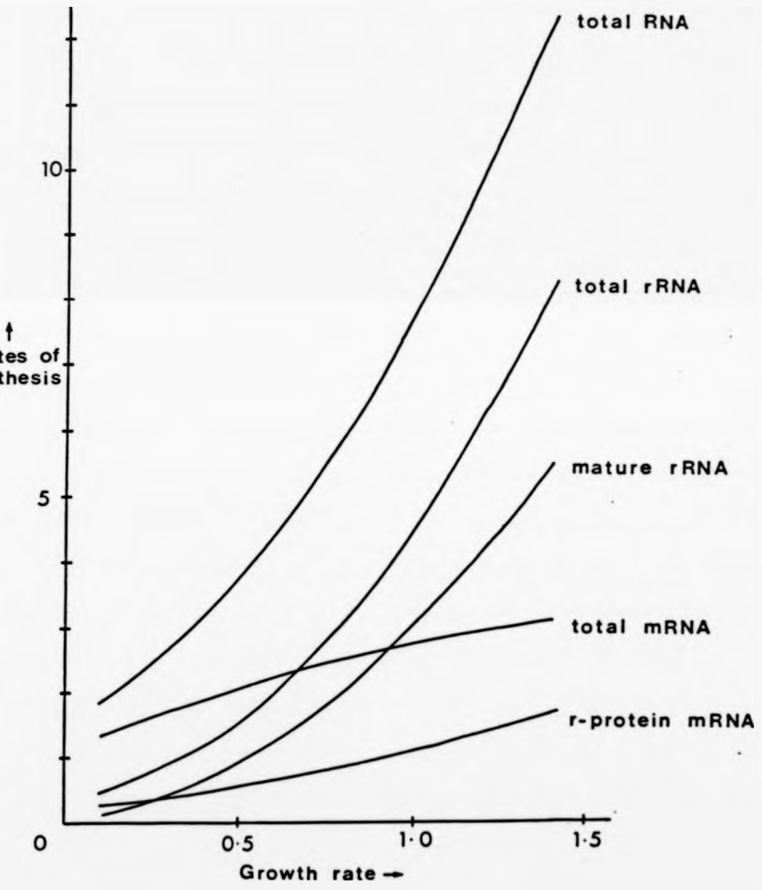
There is also evidence for transcriptional control of ribosomal protein synthesis. A number of workers have examined the quantity and relative rates of synthesis of the ribosomal components over a variety of growth rates and these data can be contradictory. However, figure 1.5 (Gausing 1977) illustrates a number of important points. 1) The rate of synthesis of mature and stable rRNA increases in proportion with the growth rate. 2) The rate of synthesis of r-protein mRNA increases with the growth rate indicating the action of a transcriptional control mechanism. 3) The rates of synthesis of rRNA and r-protein mRNA are not closely linked. 4) The increase in the rate of synthesis of the structural ribosomal components, with increasing growth rate, significantly exceeds that of r-protein mRNA; indicating that the translational efficiency from r-protein mRNA increases with increasing growth rate; ie. supports the translational feedback model. 5) The proportion of rRNA that is broken down is far greater for low growth rates (Norris & Kock 1972).

Figure 1.5.

Variation in the Rates of RNA Synthesis With Respect to Growth Rate in *E. coli*.

Rates are calulated as the weight of RNA/weight of total protein per minute. Growth rate is given as generations per hour. The rates of synthesis of r-protein mRNA have been multiplied by a factor of 10 for clarity.

Figure taken from Gausing (1977).



A number of more specific studies have been carried out to examine possible mechanisms of pre-translational control of r-protein synthesis. Sequence analysis of several r-protein operon promoter regions failed to reveal any high degree of homology. The only common feature of interest is a GC rich region between the Pribnow box and the transcriptional start site. It was therefore considered unlikely that these promoters possess the necessary homology to respond similarly to regulatory moieties. Sequence analysis of a number of r-protein genes eg. S20 (Mackie 1981, Mackie & Parson 1983), L34 (Hansen *et al* 1982) and S21 (Burton *et al* 1983) has, however, revealed the presence of a number of tandem or multiple promoters that may be found to exert differential transcriptional control.

There is evidence to suggest that the S10 operon is subject to transcriptional control. It has been reported that an over-production of L4 *in vivo* results in a marked decrease in the synthesis of mRNA from this operon (Zengel *et al* 1980). Lindahl *et al* (1983) have demonstrated that this inhibition is due to transcriptional termination at a point 30 bases upstream from the translational initiation site of the first gene. The result is a 140 base transcript. Although the mechanism of attenuation is unclear, a number of interesting observations have been made. 65 bp from the transcriptional initiation point resides a 96 bp open reading frame which unfortunately lacks a recognizable Shine-Dalgarno sequence casting doubts on its ability to be translated. On the other hand, the central region of this sequence can be represented as a 44 base stem

and loop structure followed by a run of 4 uridine residues. This structure is somewhat reminiscent of a transcriptional terminator and it was shown that the attenuated transcripts do in fact terminate at the UUUU sequence. It was further noted that the stem and loop possesses a 9 base sequence which is also found on the 23S rRNA molecule in close proximity to the L4 binding site. Though L4 binding at this stem and loop structure has yet to be demonstrated, Lindahl *et al* (1983) speculate that free L4 might bind to this putative attenuator to stabilize its otherwise unstable structure, thereby enhancing RNA chain termination. For the S10 operon, therefore, there appears to be both transcriptional attenuation and translational feed-back repression mechanisms at work (Yates & Nomura 1980). Attenuation of transcription has not been demonstrated in any other r-protein operons, but similar attenuator-like structures have been proposed for some of these operons.

1.2.5. Regulation of r-Protein Synthesis by Specific r-Protein mRNA Degradation in *E. coli*.

Closer examination of the gene dosage experiments, described earlier, showed that degradation of r-protein mRNA occurs. Elevation of the r-protein gene number leads to a corresponding increase in the rate of specific mRNA synthesis; but it was found that the over-synthesized mRNA was preferentially degraded so that the steady amount of mRNA increased to a lesser degree (Fallon *et al* 1979, Olsson & Gausing 1980, Parsons & Mackie 1983). It is worth noting that the specific

feedback inhibition of r-protein translation by repressor r-protein can take place *in vitro* without mRNA degradation (Brot *et al* 1980, Yates & Nomura 1981). It is however, possible that binding of the repressor r-protein mediates mRNA degradation *in vivo*.

There is also evidence for transcriptional feedback regulation of r-protein genes by intact ribosomes via guanosine tetraphosphate (ppGpp) which will be discussed in detail below.

1.2.6. Organisation of the rRNA Operons in *E. coli*.

In *E. coli* there are seven ribosomal rRNA operons (*rrnA* to *E*, *rrnG*, *rrnH*) as shown in figure 1.3. From each operon the 16S, 23S and 5S rRNA genes are co-transcribed in that order as a single 30S rRNA precursor. Processing of this precursor results in the synthesis of equimolar amounts of all three rRNAs, with the exception of *rrnD* that has been shown to have two 5S rRNA genes. In addition, these operons have between one and three tRNA genes in the same transcriptional unit. These tRNAs are positioned either between the 16S and 23S rRNA genes or distal to the 5S rRNA gene.

Figure 1.3 shows the operon positions to be in the same hemisphere of the chromosome as the initiation site for DNA replication *oriC*. This positioning facilitates an effective increase in operon copy number as the growth rate increases. For *E.coli*, calculations predict that

this increase is only 20% as the growth rate increases from 0.9 to 2.7 doublings per hour (Ellwood & Nomura 1982). However, rRNA synthesis is independent of gene dosage in *E. coli* (see below), but, the contribution from each operon to the total rRNA synthesis will be influenced by the gene dosage; i.e. origin proximal operons (e.g. *rrnC*) will contribute more rRNA than will origin distal operons e.g. *rrnH*).

Each rRNA operon contains tandem promoters called P1 and P2 which are separated by about 120 bases. Each promoter has recognizable -35 and -10 sequences. A strong sequence homology exists between the -10 positions of the P1 promoters which may have an important role in the regulation of rRNA gene expression (Deboer *et al* 1979). P1 is the major promoter *in vivo* with activities several times that of P2 (Deboer & Nomura 1979). In addition to P1 and P2, *rrnB* has two additional promoters, P3 and P4, situated more than 1 kb upstream of P1 and P2 (Boros *et al* 1983). Analogous promoters to P3 and P4 have not been found in *rrnD* or *rrnH* (Nomura *et al* 1984). The contribution of P3 and P4 to rRNA synthesis is not known and an open reading frame of unknown function exists between the two pairs of promoters.

1.2.7. Regulation of rRNA and Ribosome Synthesis in *E. coli*.

As stated above, transcriptional regulation of rRNA is in some way mediated by 'free' ribosomes that are in excess to the cells protein synthesizing requirement for any

particular growth rate; i.e. negative feedback regulation of rRNA transcription occurs. The effectiveness of this regulatory mechanism can be demonstrated by gene dosage experiments. The introduction of additional rRNA operons into a cell does not significantly increase the rate of rRNA synthesis (Jinks-Robertson *et al* 1983). However, it was also demonstrated that the additional rRNA genes must produce rRNA products that are capable of assembling into functional ribosomes: that is, elevation of the rRNA operon copy number with assembly defective genes results in an increase in the combined (chromosomal and defective) rate of rRNA synthesis. This result implicates the involvement of the intact ribosome. Further evidence comes from cold sensitive r-protein mutants that are defective in ribosome assembly at the non-permissive temperature. At the non-permissive temperature these mutants were shown to oversynthesize rRNA at higher rates than the parent strain at the same temperature (Nomura *et al* 1984). A lesser inhibition of r-protein gene transcription was also observed indicating the action of a weaker feedback mechanism functioning at these operons. It has been shown that P1 is the most responsive promoter to growth rate regulation and that P2 appears to be a constitutive promoter with an activity that is only significant at low growth rates or during recovery from stationary phase growth.

Nomura's group have been unable to show direct and specific regulatory effects of ribosomes on transcription from ribosomal promoters (Nomura *et al* 1984). This being the

case, the existence of effector moieties must be considered. The prime candidates are the highly phosphorylated nucleotides (HPN's) guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) and there is a great deal of evidence linking ppGpp with both stringent control and growth rate dependent control.

1.2.8. The Role of Highly Phosphorylated Nucleotides in the Regulation of Ribosome Synthesis in *E. coli*.

E. coli cells maintain a basal level of ppGpp in the region of 0.02 to 0.06 mM (Nierlich 1978). In actively growing cells an inverse relationship is found between this concentration and the growth rate of the cell and hence to the rate of ribosome synthesis. Let us therefore, consider the evidence for ppGpp mediated control and of ribosome synthesis.

Amino acid deprivation of *E. coli* brings about a so called stringent response. This state is typified by a 10 to 20 fold increase in the basal level of ppGpp in the cell with a concomitant 10 fold decrease in the transcriptional rate of the rRNA, tRNA and r-protein genes (Lamond & Travers 1985). In addition, a wide variety of additional effects are observed. These include reductions in the rate of initiation of DNA replication, the biosynthesis of carbohydrates, lipids, nucleotides, peptidoglycans and glycolytic intermediates; intra-cellular proteolysis is increased and the transport of many macromolecular precursors across the membrane is inhibited (Lamond & Travers 1985).

ppGpp has also been shown to inhibit a number of enzymes and to activate phosphoenol pyruvate decarboxylase (Gallant 1979). O'Farrell (1978) examined the rates of synthesis of over 300 different polypeptides in a comparison between cells, prior to and in the stringent response. The results showed the expression of 50% of these polypeptides to be either stimulated or inhibited. Clearly the stringent response heralds a massive adjustment of cellular function. The overall effect is to 'rein in' on potentially wasteful activities presumably to improve the cell's chances of surviving nutritional deprivation.

The synthesis of ppGpp is carried out by the *relA* gene product; the stringent factor or ppGpp synthetase I (Richtar 1976), which is found in close association with the ribosomes. Synthesis of one ppGpp molecule occurs with the binding and subsequent release of an uncharged tRNA at the ribosome A site (Richtar 1976). *relA* mutants that lack a functional stringent factor do not show elevated ppGpp levels and continue to synthesise stable RNA's during amino acid deprivation. Such mutants are termed relaxed (hence *rel*).

Other *rel* mutants are documented. *relB* mutants lead to the production of an endogenous ribosome inhibitor during amino acid deprivation which indirectly blocks stringent factor activity (Diderichsen *et al* 1977). The *relC* gene product is the L11 ribosomal protein. *relC* mutations reduce stringent factor activity on the ribosome (Parker *et al* 1976). The *relX* gene is situated 6 kb from the *relA* gene and is implicated in control of the basal levels

of ppGpp. Mutations in *relX* exhibit a four-fold drop in these levels.

ppGpp has been shown to act at the level of transcription. Studies on the structure of promoters from both stringently and non-stringently regulated genes have pointed towards three important features and it has been suggested that a stringently regulated promoter requires at least two of the three (Lamond & Travers 1985). The first is a GC rich 'discriminator' at, or close to, the transcriptional start site (Travers 1980). The second is a -35 sequence that deviates from the highly conserved TTGA sequence. The third is a putative RNA polymerase binding site situated at position -40 to -98 (Lamond & Travers 1983). The -10 region provided no features of interest.

Travers *et al* (1983) have proposed that ppGpp influences the selectivity of RNA polymerase towards stable RNA promoters. This model suggests that only one particular form of the RNA polymerase (probably a dimer) is able to transcribe stable RNA promoters and that ppGpp can inhibit such a conformational change. There is no *in vivo* evidence that ppGpp interacts directly with the RNA polymerase. However, a relaxed phenotype has been documented for RNA polymerase mutants that continue to synthesis ppGpp in response to amino acid deprivation (Nene & Glass 1983).

Unfortunately, there are exceptions to the above clear cut portrayal of the role of ppGpp. There are reports describing cases in which amino acid deprivation has led to a reduction

in rRNA synthesis but no increase in ppGpp being observed (Spadaro *et al* 1981, Belitsky & Kari 1982). Conversely, in an EF-G mutant of *B. subtilis*, amino acid deprivation was terminated by a temperature shift up resulting in an increase in rRNA synthesis but no decrease in the ppGpp concentration (Kimura *et al* 1974).

In general the levels of pppGpp mimics that of ppGpp but at a several fold lesser concentration. Probably for this reason, studies in this field have concentrated on ppGpp. A third HPN, ppGp, has also been described (Pao & Gallant 1979, Pao *et al* 1979) and was shown to preferentially inhibit rRNA and tRNA synthesis. In addition, a variety of other HPN's have also been reported in *B. subtilis* (see below) (Rhaese & Groscurth 1976).

1.3. DNA Methylation.

Eukaryotic DNA is known to contain the modified base 5-methylcytosine (5mC). In addition to 5mC, prokaryotic DNA can also contain 6-methyladenine (6mA), though some strains of bacteria have been shown to possess no detectable 5mC or 6mA (Wachsman & Irwin 1970, Schein *et al* 1972). The base 4-methylcytosine has been reported in a number of thermophilic bacteria (Ehrlich *et al* 1985) and a variety of modified bases have been documented for bacteriophage (Hatman 1981). The level of the modified bases are shown to vary from strain to strain, but patterns of DNA methylation are heritable and are maintained by DNA methyltransferase in an early post-DNA-replication step

(Burden & Adams 1969). It has also been shown for *E. coli* at least, that the methylation pattern is ubiquitous for all DNA's within a cell (Razin *et al* 1980)

A number of methods are available for the study of modified bases in DNA. These include chromatographic procedures, electrophoretic separation, gas chromatography, mass spectrometry and high pressure liquid chromotography (Doerfler 1983). Small quantities can be detected by raising antibodies to 5mC that has been covalently bound to bovine serum albumen (Sano *et al* 1980). More detailed information can be derived from differential restriction endonuclease sensitivity (Bird & Southern 1978) and Maxam and Gilbert sequencing (Nick *et al* 1986). The cytidine analogue 5-azacytidine, which cannot be methylated by the cell, can also be utilized to probe the effects of under methylation (Creusot *et al* 1982).

1.3.1. Regulation of Cellular Processes by DNA Methylation.

There is no doubt that the modification of DNA bases can markedly influence the interaction between DNA and proteins. In prokaryotes the interference of restriction endonuclease activity by specific methylated bases is clearly documented. However, in the eukaryotes there is overwhelming evidence that 5mC can markedly influence gene expression. The best evidence comes from microinjection of cloned DNAs into *Xenopus laevis* oocytes or mammalian cells. These studies verify that a gene methylated at a specific position can be transcriptionally inactive (Vardimon *et al* 1982). Of

particular interest to the subject of this thesis is the control of rRNA genes by DNA methylation. There are numerous publications correlating transcriptional activity of rDNA genes with their state of methylation in a number of eukaryotes, e.g. mice (Bird *et al* 1981b) and *X. laevis* (Bird *et al* 1981a). In prokaryotes, however, evidence for the regulation of gene expression by DNA methylation is limited and centres largely on *E. coli*.

In *E.coli* the modification methylases account for only a fraction of the total 6mA activity. The major adenenine methylase activity is encoded by the *dam* gene. In the absence of the *dam* methylase less than 1% of the 6mA normally found in DNA remains. The *dam* methylase specifically recognizes the sequence 5'-GATC-3'. A general idea that appears to be emerging from the studies on the biological role of 6mA is that it can couple gene expression to DNA replication and cell division if the gene is transcribed poorly from fully methylated DNA and efficiently from hemi-methylated DNA (Sternberg 1985). These genes may then be expressed during the period between DNA replication and the subsequent methylation of the newly formed strand.

dam methylation is known to mediate gene regulation in the transposons Tn10, Tn903 and Tn5 (Kleckner *et al* 1984). Tn10 is the best studied and exhibits the most striking relationship with *dam*. Tn10 transposition is elevated 10 fold and Tn10 mediated deletion or inversion is elevated 100 to 250 fold in *dam*$^-$ cells. Tn5 and Tn903 show 7 to 10 fold elevation of transposition in *dam* $^-$ cells.

dam methylation has been shown to act in two ways (Roberts *et al* 1985). The first is by inserting 6mA in the -10 (GmATCAAAT) region of the transposase gene. This modified base reduces transcription from this promoter by 5 to 10 fold. Secondly, 6mA situated near the ends of the transposon inhibit their interactions with the transposase.

An ingenious method was used to study the effect of hemimethylated DNA on Tn10 transposition (Roberts *et al* 1985). Hfr DNA containing Tn10 was transferred from a *dam*$^+$ strain in single stranded form to a *dam*$^-$ recipient. In the *dam*$^-$ background the newly synthesized complementary strand was unmethylated. This new hemi-methylated DNA remains in the cell until it is either degraded or intergrated into the host chromosome by homologous recombination or transposition. Transposition of hemimethylated transposons was shown to be increased by up to 1000 fold. The suggestion is that transposition events are most likely to occur immediately following DNA replication.

Methylation of promoter sequences has also been shown to reduce transcription by 2 to 6 fold from *trpR* (Marinos 1985), *sulA* and bacteriophage P1 *cre* (see Sternberg 1985). All of these genes contain *dam* methylation sites in the -35 region of their promoters. A similar observation has been made in the arabinose operon (see Doefler 1983). The *lac* repressor has also been shown to bind more tightly to a mutant operon containing a 5mC (Fisher & Caruthers 1979).

The Mu *mom* gene encodes a DNA modification function. The phenotype requires the expresion phage *mom* gene and the host *dam* gene (Toussaint 1977). Hattman (1982) has shown that *mom* regulation operates at the transcriptional level since the rate of *mom* transcription is reduced 20-fold in *dam*$^-$ host. The *mom* promoter has no *dam* sites but 3 are located just uptream (Hattman & Ives 1984). Removal of these *dam* sites eliminates *dam* dependency.

Studies on the role of *dam* methylation in the initiation of DNA replication are also very interesting. *oriC* contains 12 to 14 *dam* methylation sites within its 250 base pairs (Oka *et al* 1980). Conservation of 8 of these *oriC dam* sites was found in five Gram negative organisms (Zyskind *et al* 1983). Plasmids based on *oriC* for replication transform *dam*$^-$ hosts poorly or not at all. In addition, *oriC* DNA isolated from a *dam*$^-$ background functions 2- to 4-fold less well than their *dam*$^+$ counterparts in an *in vitro* initiation system (see Sternberg 1985). Finally, increasing *dam* gene copy number on multicopy plasmids, and hence *dam* methylase levels, results in the reduction of the spacing between initiation events of DNA relication (Messer *et al* 1985). This final result has lead to the suggestion that the spacing between initiations of DNA replication is influenced by the time required to methylate the newly synthesized strand of *oriC*. It is of further interest that even in *dam*$^-$ mutants some methylation of *oriC dam* sequences still occurs (Smith *et al* 1985). The identity of this methylase

activity awaits elucidation. It has been shown that 6mA-T base pairs are more readily denatured than A-T base pairs (Engel & von Hippel 1978). *oriC* methylation may, therefore, ease strand separation at *oriC* facilitating initiation of DNA replication.

1.4 Regulation of Cellular Processes by DNA Reorganisation.

A number of cases have been described in prokaryotes where physical rearrangement of the chromosome alters gene expression. Indeed, one such rearrangement occurs concomitant with prokaryotic development ie. during *Anabaena* 7120 heterocyst formation (Golden *et al* 1985). In general, such rearrangements and transpositions involve the presence of either direct or inverted repeat sequences (DRS or IRS respectively) which are recognized and utilized by transposases or invertases, or simply used in genetic recombination. IRS can also functions as sites for protein recognition, initiation of DNA replication, termination of transcription and RNA processing.

Interestingly, differentiating organisms appear to have a greater proportion of inverted repeated sequences (IRS) than do organisms with less complex cell cycles. The following percentages of IRS have been reported, 0.5% in *E. coli* (Kato *et al* 1974), 5% in *B. subtilis* (Galloway & Rudnor 1979), 5% in *C. crescentus* (Woods *et al* 1976) and 7% in *R. vannielii* (Russel & Mann 1986). In addition, cell cycle asociated DNA rearrangements have been reported for *C. crescentus* (Nisen *et al* 1979) and *B. subtilis*

(Rhaese *et al* 1982). However, there is no evidence to show that DNA rearrangements can be responsible for prokaryotic differention, though it is tempting to speculate.

Halobacteria have been shown to have a large number of repeated sequences in greater than fifty families. Rearrangements associated with these repeated sequences have been shown to occur at very high frequencies (Sapienza *et al* 1982).

The phase variation of *S. typhimuriumum* is the best known example of regulation of gene expression by DNA inversion (Zieg *et al* 1977). Phase variation refers to the variability of major antigenic determinant on the cell, the flagellum. There are two distinct flagellin genes in *S. typhimurium*, H1 and H2, but only one is transcribed at a time, see figure 1.6.a. The invertible H region is 970 bp in length and is flanked by 14 bp inverted repeat sequences. It contains the *hin* gene with its promoter and a second promoter that initiates transcription near the end and away from this invertible region. The *hin* gene product (the invertase) is responsible for the inversion event through interaction with the IRS (Silverman & Simon 1980).

In the 'H2 state' the outward facing promoter transcribes both the H2 and the rH1 gene. The rH1 gene encodes an H1 gene repressor, that blocks H1 gene transcription (Fujita *et al* 1973). The H1 state is initiated by the inversion of the H region such that the outward facing promoter can no longer initiate transcription of H2 or rH1. Without the

Figure 1.6.

Regulation of Gene Expression by DNA Rearrangement.

In each figure the invertible region is flanked by IRS's which are identified by closed boxes. The orientation of genes is indicated by a straight arrow, with transcriptional activity from promoters (P) being indicated by a wavey arrow. Refer to text for detailed explanations.

Figure 1.6.a.

Genetic Basis for Phase Variation in *S. typhimurium*.

H1 and H2 are two alternative flagellin genes, with rH1 being a repressor of H1 transcription. *hin* is the invertase gene.

Figure 1.6.b.

Invertable G Region of Phage Mu.

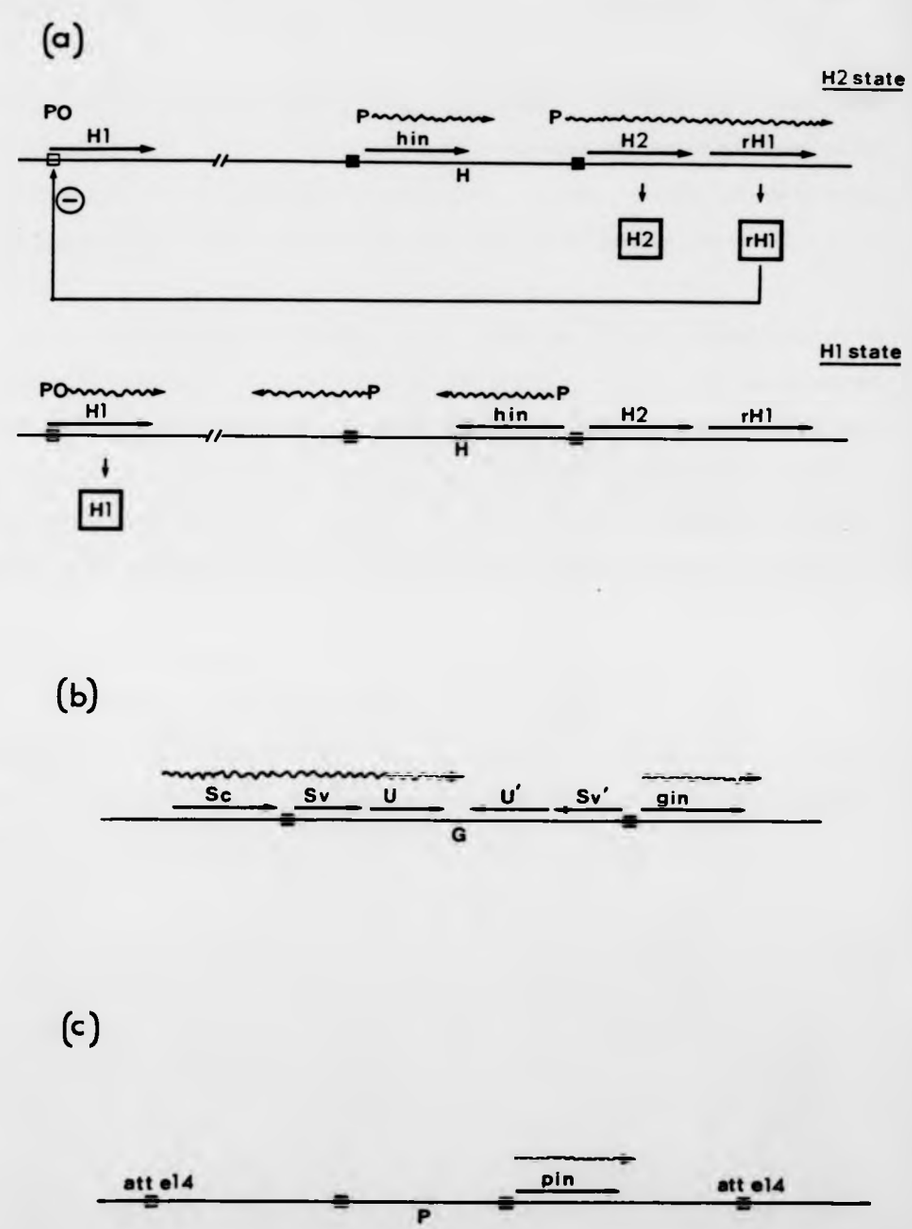
S and U gene products are involved in the assembly of the phage tail fibres. Sc (S constant) and Su (S variable) are co-transcribed to form a complete S gene product. *gin* is the invertase gene.

Figure 1.6.c.

e14 Element of *E. coli* K-12.

pin is the invertase gene.

Figures taken from Plasterk and van de Putte (1984).



presence of its repressor, H1 may now be expressed.

An interesting story is currently emerging for the antigenic variation shown by *Neisseria gonorrhoeae* pili where a system of DNA rearrangements reminiscent of antibody gene variations has been proposed (Haas & Meyer 1986).

Mu is a temperate phage that has a 3 kb invertible G segment flanked by a 34 bp IRS (Figure 1.6.b). Inversion of this region facilitates recognition of a different host range by the phage (van de Putte *et al* 1980). The genes S and U encode products that are involved in the assembly of tail fibres and consequently influence the host ranges. Phage Mu can either express the genes S and U or their counterparts S' and U' depending upon the orientation of the G region (Howe *et al* 1979). Transcription is initiated from 500 bp outside the G region and this represents a common sequence (Sc) to both S and S', that are completed by the variable Sv and Sv' regions, respectively (Giphart *et al* 1982).

The DNA inversion of Mu differs from that of *S. typhimurium* in a number of aspects. In the case of Mu, neither the 'regulatory promoter' nor the transposase gene (*gin*) is situated on the invertible region and in addition a gene fusion event occurs.

The phage P1 has an invertible C region that shows striking similarities to the G region of Mu.

The *E. coli* K-12 genome contains a 14 kb element called

e14 (Greener & Hill 1980) that exhibits the properties of a defective prophage. It can be excised from the chromosome following UV irradiation but cannot replicate autonomously. Within e14 is a 1794 bp invertible P region (Plasterk & van de Putte 1985)(Figure 1.6.c). The invertase gene (*pin*) is situated adjacent to the P region. As yet no function has been assigned to the P region.

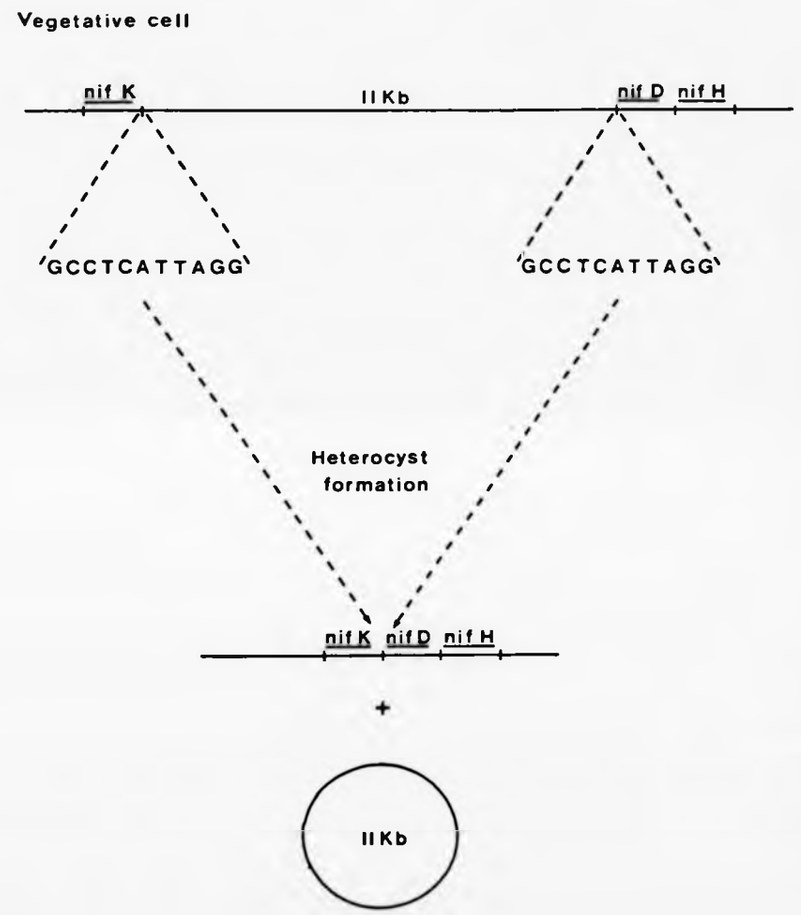
It is of interest to note that a striking homology is observed both structurally and functionally between the *hin*, *gin*, *cin* (phage P1 invertase) and *pin* gene products.

Heterocyst formation in *Anabaena* 7120 is triggered by fixed-nitrogen deprivation. A non-nitrogen fixing vegetative cell develops into a specialized nitrogen fixing heterocyst. This transformation is accompanied by a change in the DNA structure in the vicinity of the three nitrogenase structural genes, *nifK*, *nifD*, and *nifH* (Golden *et al* 1985). In the vegetative cell the *nifK* gene is separated from *nifD* and *nifH* by 11 kb as shown in figure 1.7. This 11 kb region is flanked by 11 bp DRS and is excised during heterocyst formation. In so doing the equivalent of 23 amino acids are lost from the original *nifD* gene and are replaced by the equivalent of 43 new amino acids. The excised 11 kbp region remains within the heterocyst as a covalently closed circle. This genomic rearrangement is presumably necessary for functional expression of the nitrogenase genes.

Figure 1.7.

Rearrangement of Nitrogenase DNA During Heterocyst Formation in *Anabaena* 7120.

The genes *nifK*, *nifD* and *nifH* encode the structural polypeptides for the enzyme nitrogenase. In the *Anabaena* vegetative cell the *nifK* and *nifD* genes are separated by 11kb, but are brought together during heterocyst formation by the excision of this 11kb region.



Of further interest to the subject of this thesis, Hill and Harnish (1981) have shown that homologous recombination between ribosomal genes can occur in *E. coli*. Inversions were described between *rrnD* and *rrnE* that involve about 20% of the *E. coli* genome These rearrangements were found to have little effect opon growth rate.

1.5 Sporulation in *Bacillus subtilis*.

B. subtilis is a Gram positive rod—shaped organism. During vegetative growth this cell elongates and divides by symmetric transverse binary fission to produce two equal progeny. However, under conditions of carbon and/or nitrogen limitation sporulation may be induced. This process represents a deviation from the normal vegetative process and is characterized by asymmetric division, to form two compartments with divergent developmental fates. The smaller compartment becomes the endospore and the larger compartment is destined to lyse, having first assisted in spore formation. Sporulation takes 6 to 8 hours at 37^{o}C and can be divided into seven morphological stages designated I to VII (figure 1.8).

The process of sporulation represents a complex sequence of physiological and morphological changes but not all of these are directly connected to the sporulation process, e.g. the secretion of exoenzymes, proteases and antibiotics. In addition, the induction of sporulation is triggered by an environmentally stressful event which can lead to further variations that are not directly associated with the

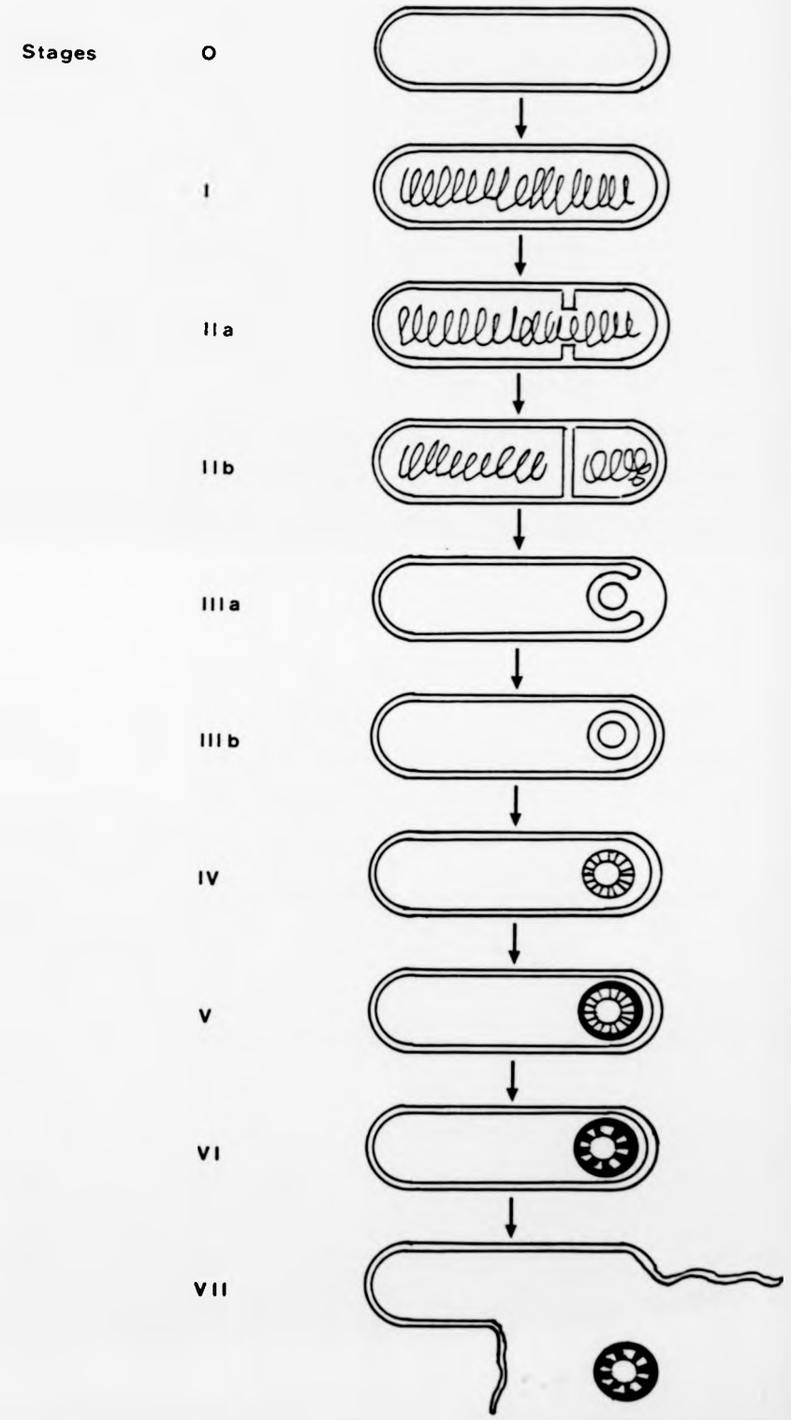
Figure 1.8.

Sporulation in *B. subtilis*

The process of sporulation in *B. subtilis* has been divided into seven stages:

Stage 0	Vegetative cell.
Stage I	Axial filament formation.
Stage II	Forespore septum formation.
Stage III	Forespore engulfment.
Stage IV	Cortex deposition.
Stage V	Coat deposition.
Stage VI	Cortex maturation.
Stage VII	Spore release.

Figure taken from Russell (1984).



sporulation process (see below).

Two facets of *B. subtilis* sporulation have been subjected to extensive examination. The first is the role of RNA polymerase sigma factor heterogeneity (Losick & Youngman 1984). At least 5 sigma factors have been described; one of these is exclusive to sporulating cells and has been shown to impose selectivity upon the RNA polymerase in the transcription of vegetative and sporulation genes (Haldenwang *et al* 1981). The second area of study is the characterization of sporulation genes; ie those genes required for the process of sporulation but having little or no function during vegetative growth. Some 50 *spo* loci have now been mapped on the *B. subtilis* genome, each representing one or more genes.

Of particular interest here, is the somewhat controversial role of highly phosphorylated nucleotides during sporulation in *B. subtilis*.

1.5.1. The Role of Highly Phosphorylated Nucleotides in *B. subtilis* Sporulation.

The role of the highly phosphorylated nucleotides (HPN's) ppGpp and pppGpp in the stringent response of *E. coli* has been already described and an analogous role for these HPNs has also been found in *B. subtilis* (Nishino *et al* 1979). However, a 'veritable zoo' of HPN's have been described for this organism during the initiation of sporulation and hence during nutrient deficiency. Rhaese

et al (1976) implicated the following HPN's with the onset of sporulation; ppApp, pppApp, pppAppp and ppZpUp (where A is adenosine, U is uridine and Z is an unidentified sugar residue: the orientation is 5' to 3'). However, Nishino *et al* (1979) failed to verify the presence of pppAppp and more importantly, showed that the sporulation process was not impaired in a relaxed mutant (Swanton & Edlin 1972) that was unable to accumulate any HPN's. It is now generally accepted that HPN accumulation in *B. subtilis* is only implicated with the nutritional status of the cell and not with the process of sporulation.

Alternatively, Freese and his co-workers (eg. Ochi *et al* 1982) have argued that sporulation is in fact caused by a decrease in GTP or GDP. They have shown that reduction in the biosynthesis of these nucleotides either by mutations or by specific enzyme inhibition causes an induction of sporulation, even in the presence of otherwise repressing levels of nutrients.

1.6. Cell Cycle of *Caulobacter crescentus*.

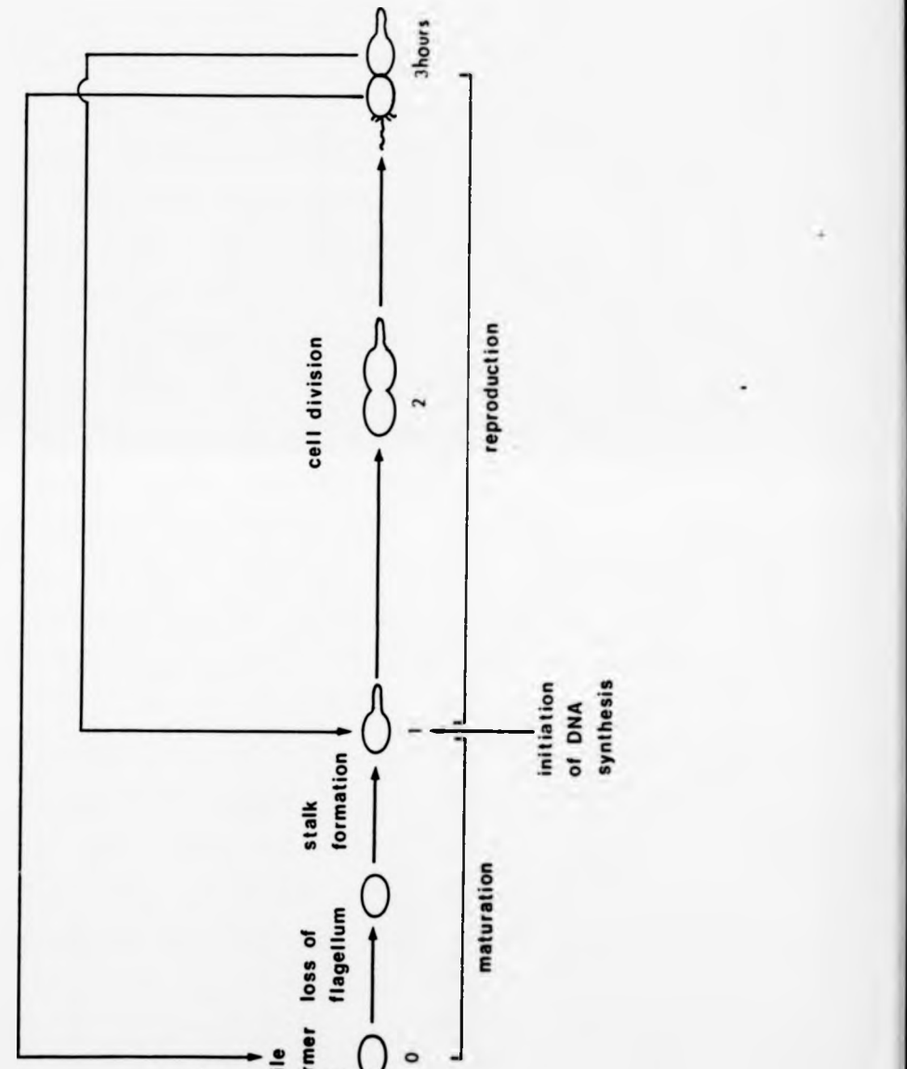
The cell cycle of *C. crescentus* (figure 1.9) is well worth consideration as it exhibits many similarities with the simplified cell cycle of *R. vannielii* and contrasts significantly with that of *E. coli*. In addition, it is a well studied system.

The cell cycle of both *C. crescentus* and *R. vannielii* are taken to begin with the motile swarmer cell. The swarmer

Figure 1.9.

The Cell Cycle of *C. crescentus*.

The cell cycle of *C. crescentus* is taken to begin with the motile swarmer cell. Cell maturation is characterized by the loss of the flagellum and the subsequent synthesis of a stalk. Cell division is by asymmetric binary fission to yield two unequal cells with differing cell cycle destinies.



cell of *R. vannielii* is peritrichously flagellated whereas that of *C. crescentus* exhibits visible polarity with the possession of a single flagellum and pili at one cell pole; it is from this same pole that the stalk will be formed. The physiological state of the swarmer cells is of great importance. The swarmer cell is unable to reproduce *per se* and does not replicate its DNA. These cells must undergo a period of maturation to gain the capacity for reproduction. In addition, the *R. vannielii* swarmer cell (grown photoheterotrophically under anaerobic conditions) can only initiate maturation when sufficient light is supplied to trigger the process; i.e., this viable cell will remain shut down with respect to growth until it receives the required light stimulation (see chapter 1.8.1). For *Caulobacter* an analogous situation exists in as much that in chemostat experiments, carbon limitation prolongs the swarmer cell stage of the cell cycle. A survival/dispersal role has been assigned to this growth precursor cell (Dow *et al* 1983).

In both systems maturation proceeds with the loss of the flagella and the development of a stalked (*Caulobacter*) or prosthecate (*R. vannielii*) adult cell. In *R. vannielii* reproduction is by the formation of a progeny cell at the tip of the prosthecae, whereas the stalk of *Caulobacter* is not directly involved in the process of cell division. In both cases, however, cell division is asymmetric yielding both a progeny cell and an ageing parental cell, each with differing cell cycle destinies. The parent cell is able to immediately initiate a new round of replication, where as the progeny cell must first complete a period of maturation

before replication can occur.

In *C. crescentus* differential expression of a number of cellular components has been described (Ohta *et al* 1985, Gomes & Shapiro 1984). In contrast, rDNA has been isolated and well characterized (Ohta & Newton 1981, Feingold *et al* 1985), but no evidence for cell cycle associated modulation of synthesis has been proposed. However, it should be emphasized that a direct comparison cannot be made between the *Caulobacter* swarmer cell and the inhibited *R. vannielii* swarmer cell that is incubated anaerobically in the dark. Unfortunately, it is not experimentally feasible to remove the energy source from *Caulobacter* swarmer cells to achieve a fully inhibited state.

1.7. *Rhodomicrobium vannielii*

R. vanneilii was first isolated in 1949 by Duchow and Douglas (1949). It is a member of the Rhodospirillaceae and is capable of growing both photoheterotrophically under anaerobic conditions in the light or chemoheterotrophically under aerobic conditions in the dark. Photoheterotrophic growth is routinely used in the laboratory. This Gram negative organism is characterized by ovoid cells (2-3 μm by 1 μm) during vegetative growth. *R. vanneilli* displays a polymorphic cell cycle (Whittenbury & Dow 1977) and a typical laboratory culture will be found to contain several distinct cellular morphologies together with a variety of multi-cellular configurations.

1.7.1. The Cell Cycle of *R. vannielii*.

Figure 1.10 details the essential features of the *R. vannielii* cell cycle which is taken to begin with the peritrichously flagellated swarmer cell. This growth precursor cell (Dow *et al* 1983) is a specialized cell that is incapable of replication *per se*. It does not carry out DNA replication (Potts & Dow 1979) and protein synthesis is much reduced (Porter 1984) presumably to a 'maintenance level'. Further, during anaerobic photoheterotrophic growth this active cell will remain as such until provided with sufficient light to 'trigger' the process of maturation. For these reasons a dispersal or survival role has been assigned to the swarmer cell (Dow *et al* 1983): that is, in moderately adverse environments this highly motile cell will be unable to initiate maturation until it finds a new and suitable environment. This environmental switch to initiate swarmer cell maturation is also beneficial in the laboratory as swarmer cells can be induced to develop synchronously by providing illumination.

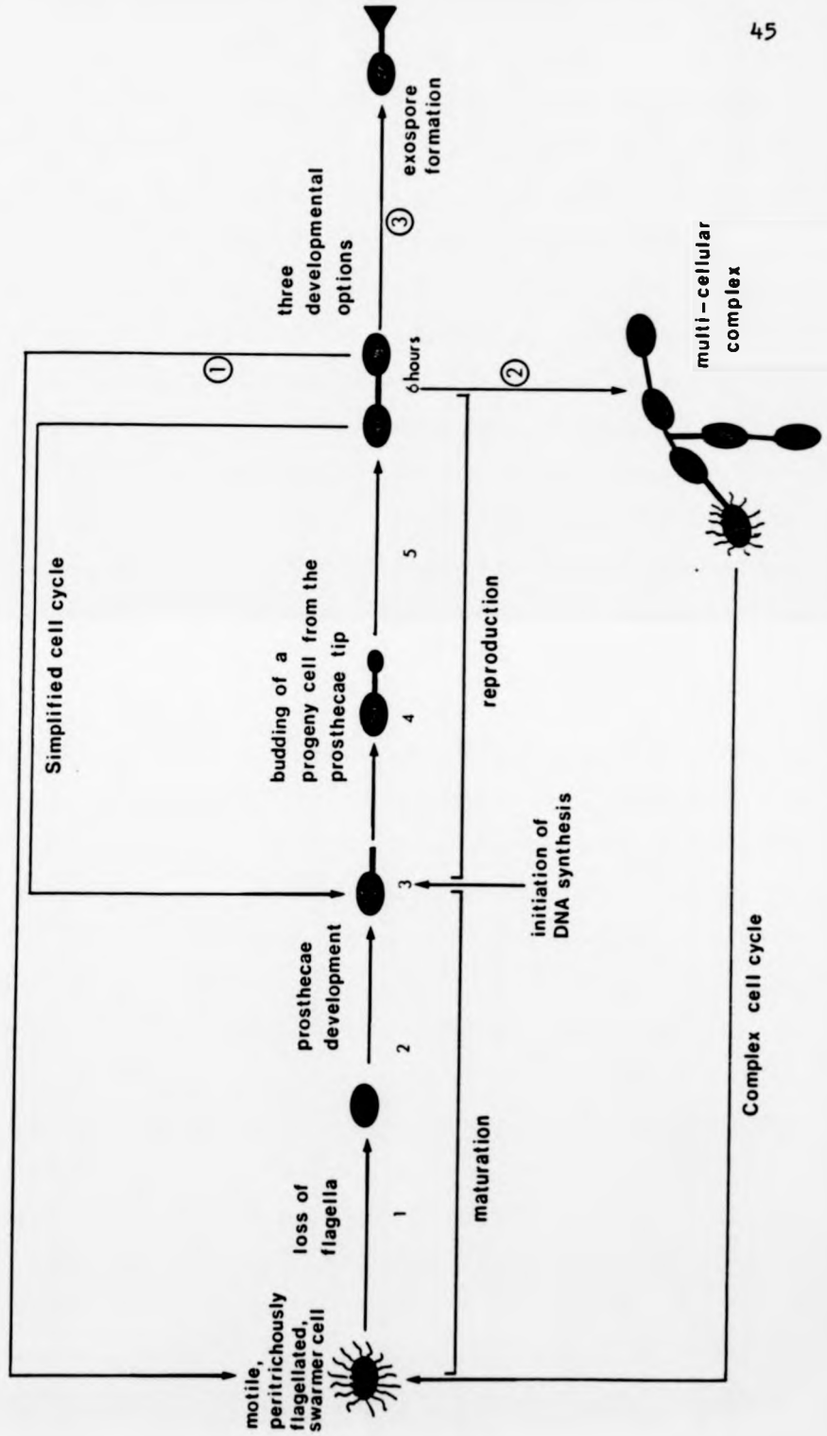
Swarmer cell maturation is characterized by loss of flagella and the development of a prosthecae from one of the two cell poles i.e.. polar growth. This maturation process involves a controlled series of distinct molecular events that can only be carried out once by any given swarmer cell and are irreversible.

The prosthecate or 'mother' cell will now undergo a process of asymmetric division. A new 'daughter' cell is

Figure 1.10.

The Cell Cycle of *R. vannielii*.

The cell cycle of *R. vannielii* is taken to begin with the motile swarmer cell. Cell maturation is characterized by the loss of the flagella and the subsequent synthesis of a prostheca. A progeny cell is formed from the prostheca tip and will embark upon one of three developmental options : swarmer cell formation with physical separation, chain cell formation or exospore formation.



formed at the distal end of this integral cellular extension, the prostheca. The new cell has three developmental 'options'. The first to become a swarmer cell which, when complete, will separate from its mother by binary fission. During photoheterotrophic growth swarmer cell formation is favoured in situations of low light and high CO_2 tension (25ml l^{-1}). Constitutive swarmer cell formation is described as the simple cell cycle (Dow & France 1980). Once cell separation has occurred, the mother cell is free to produce a new daughter cell. It is clear that the mother cell undergoes an ageing process and exhibits signs of mortality in only being able to produce a maximum of four daughters, irrespective of their cell type (Whittenbury & Dow 1977).

The second option is to become a chain cell which remains attached to the mother's prostheca. Cell division is effected by the formation of a plug within the prostheca. This plug is thought to physically separate the cells, but has been shown to contain a central pore (Dunham, personal communication) of unknown function. This chain cell must now undergo a limited maturation which is characterized by development of its own prostheca. During photoheterotrophic growth the formation of a chain cell is favoured by higher light intensities and low CO_2 tensions. Repetition of this process with branching of existing filaments results in the formation of the characteristic multicellular complexes of *R. vannielii.*

The third option is to become an angular, pyramidal

exospore. Exospore formation is favoured by nutrient depletion as encountered in the stationary phase of batch culture. These exospores have been shown to be resistant to both heat and dessication but not to UV inactivation or lysosyme activity (Whittenbury & Dow 1977). Germination occurs on nutrient replacement.

1.7.2. Why Study *R. vannielii*?

There are a number of aspects that make *R. vannielii* a very attractive candidate for the study of cell type expression control and the concomitant control of gene expression in prokaryotes. These include the possession of a characteristic developmental path with an abundance of 'landmark events', that are readily observable by phase contrast microscopy and cell size distribution analysis. Swarmer cells can be rapidly and reproducibly selected from large culture volumes (Whittenbury & Dow 1977). The researcher can exert control over the initiation of swarmer cell differentiation via illumination. Finally the differentiation of the swarmer cell is a natural element of *R. vannielii's* vegetative growth.

1.7.3. Background to *R. vannielii* Research.

Inevitably, due to the ease of swarmer cell selection and synchronization, the majority of studies have centered upon the maturation and initial reproduction of these cells.

Unlike the situation of *E. coli* (Lutkenhaus *et al* 1979),

qualitative and quantitative changes in protein synthesis have been shown for both soluble proteins (Porter 1984) and intra-cytoplasmic membrane proteins (Kelly 1985) during swarmer cell maturation and reproduction. A number of these polypeptides have been identified. The first is the Mr 34,000 flagellin monomer which is only synthesized in the swarmer cell (Kelly 1985). A second group of proteins comprise the B885-RC complex (Kelly 1985) which were shown to be synthesized during the reproduction stage as defined in Figure 1.10. An additional Mr 11,500 protein has been extensively studied. This polypeptide was shown to have a very rapid turnover, as indicated by ^{35}S-methionine labelling (Porter 1984). It is present in the swarmer cell but is actively broken down during swarmer cell maturation. Unfortunately, the function of this polypeptide is still unknown.

Studies have been carried out using ^{3}H-DFP (di-isopropylfluoro phosphate) (Russell & Mann 1984) which covalently interacts with the serine residue in the active site of serine proteases. Quantitative differences in autoradiograph banding patterns are apparent during swarmer cell maturation and reproduction, together with the appearance of a Mr 57,000 band near the time of daughter cell completion. Such variations may reflect either enzyme quantity or the availability of the active site (Russell & Mann 1984).

Whittenbury & Dow (1977) showed the swarmer cell nucleoid to be in a condensed format and located at the centre of the

cell. Upon initiation of differentiation the nucleoid was observed to elongate and migrate to the pole of the cell from which the prostheca will form. The nucleoid returned to the centre of the cell at a time when the daughter cell was roughly half the size of the mother. It was proposed that this configurational change in the nucleoid may play a role in control of gene expression during swarmer cell maturation (Dow *et al* 1983).

The role of RNA polymerase has been considered in the modulation of gene expression during the differentiation of *R. vannielii*. The core enzyme is similar in subunit size and composition to that of *E. coli*. Non-denaturing gel electrophoresis has shown three further polypeptides (Mr 70,000, 80,000 & 95,000) to be associated with this core. Scott & Dow (JGM, in press) have proposed that quantitative changes that occur in these putative sigma factors during differentiation, may play a role in gene selection.

The *R. vannielii* genome is 2.1×10^9 daltons in size with a G + C content of 62.5% (Potts *et al* 1980). In addition it has been shown to have about 7% inverted repeat sequences (IRS) (Russell & Mann 1986). These IRS were found in two size classes of 100 to 700 bp and 17 - 27 bp which appear to be scattered throughout the chromosome. No developmental specific function has yet been assigned to this large quantity of IRS.

Arguably the most important line of research in *R. vannielii* involves developing specific mutagenesis and

gene transfer techniques. Until recently, this organism appeared refractory to all endeavours in this area, but it has recently become possible to readily isolate photosynthetic mutants using essentially conventional ultra-violet (UV) and N-methyl-n'-nitro-N-nitroguanidine (NTG) mutagenesis techniques (Bredon, personal communication). In addition, promising results have also been obtained with transposon mutagenesis, showing that *R. vannielii* is competent in conjugation.

Chapter 2

Materials and Methods

2.0 Materials and Methods.

2.1 Strains.

Rhodomicrobium Vannielii strain 5 (Whittenbury & Dow 1977)

Escherichia coli DH1 : F^-, *recA1*, *endA1*, *gyrA96*, *thi*-1, *hsdR17*(r^-,m^+), *supE44*, (Hanahan 1983).

Escherichia coli MRE600 : A gift from U. Swoboda (Swoboda *et al* 1982a)

2.2 Media

2.2.1 Pyruvate-Malate Medium (PM) (Whittenbury & Dow 1977)

Ammonium chloride (NH_4Cl)	0.5 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.4 g
Sodium chloride (NaCl)	0.4 g
Calcium chloride ($CaCl_2.2H_2O$)	0.05 g
Pyruvic acid, sodium salt type II (Sigma)	1.5 g
Sodium malate	1.5 g

Made up to 1 litre with distilled water.

The medium was adjusted to pH 6.9 with potassium hydroxide. Solid media was prepared by the addition of 1.5 % (w/v) Difco Bacto Agar. Sterilization was carried out by autoclaving at 121^{o}C for 15 minutes. Sterile phosphate buffer was added aseptically to media at 45^{o}C to give a final concentration of 5.0 mM.

Phosphate Buffer.

This was prepared by mixing molar solutions of sodium di-hydrogen phosphate and di-sodium hydrogen phosphate to pH 6.9.

2.2.2 LB (Luria Bertani) Media.

Bacto tryptone	10.0 g
Bacto yeast extract	5.0 g
NaCl	10.0 g
Made up to 1 litre with distilled water	

The medium was adjusted to pH 7.5. Solid media was prepared by the addition of 1.5 % (w/v) Difco Bacto Agar.

2.2.3 Modified Davis & Mingioli Low Phosphate Minimal Media

Tris	6.055 g
Ferric chloride ($FeCl_3$)	10 ml of $0.0325\ g\ l^{-1}$
Casamino acids	0.02 g
Potassium dihydrogen phosphate (KH_2PO_4)	2.7 g
Potassium chloride (KCl)	10 ml of $0.149\ g\ l^{-1}$
Sodium citrate($.2H_2O$)	0.5 g
Magnesium sulphate ($MgSO_4.7H_2O$)	0.1 g
Ammonium sulphate $(NH_4)_2SO_4$	1.0 g
Made up to 1 litre with distilled water	

This medium was adjusted to pH 7 with hydrochloric acid and autoclaved at 121°C for 15 minutes. Once cool, 20 ml of sterile 10 % (w/v) glucose and 5 ml of trace elements solution was added aseptically.

Trace Elements Solution.

$FeSO_4.7H_2O$	0.5 g
$ZnSO_4.7H_2O$	0.5 g
$MnSO_4.3H_2O$	0.5 g
H_2SO_4 (0.1m)	10 ml

Made up to 1 litre with distilled water and autoclaved at 121°C for 15 minutes.

2.3 Solutions/Buffers.

2.3.1 TE = 10 mM Tris HCl pH8
1 mM (Na_2) EDTA pH8

2.3.2 STE = TE + 50 mM NaCl

2.3.3

SSC x 20	NaCl	175.3 g
	Sodium citrate	88.2 g

The buffer was adjusted to pH7 with 10 M NaOH and made up to a total volume of 1 litre with distilled water

2.3.4 Solutions for Ribosome Isolation.

	Solution I	Solution II
Tris HCl pH7.5	10 mM	10 mM
NH_4Cl	30 mM	30 mM
$MgCl_2$	10 mM	0.30 mM
β-mercaptoethanol	6 mM	6 mM

2.3.5 Solutions for Caesium Chloride Gradient Isolation of RNA

Solution A	Tris HCl pH8	0.1 M
	(Na_2) EDTA pH8	0.01 M
	Aurin tricarboxylic acid (ATA)	5.0 mM
Solution B	Tris HCl pH8	0.1 M
	(Na_2) EDTA pH8	0.01 M
	Caesium chloride	5.7 M
	ATA	5.0 mM

2.3.6 Electrophoresis Buffer for DNA Gels.

TBE X 5 stock	Tris base	54 g
	Boric acid	27.5 g
	(Na_2) EDTA	4.65 g

Made up to 1 litre with distilled water.

2.3.7 Buffers for RNA Electrophoresis in Agarose/Formamide Gels.

E Buffer	Tris base	43.5 g
	$NaH_2PO_4.2H_2O$	60.84 g
	(Na_2) EDTA	3.7 g
	Distilled water	1 litre

Incomplete (Gel Loading Buffer) GLB.

	E Buffer	1.2 ml
	Distilled water	3.8 ml
	Ficoll 400	0.9 g
	Bromophenol blue	

Incomplete GLB was stored in 100 µl aliquots and completed by the addition of 500 µl freshly deionized formamide.

2.3.8 Nick Translation Buffer (NTB) x 10.

Tris HCl pH7.2	0.5 M
$MgSO_4$	0.1 M
Dithiothreitol	1.0 mM
Bovine serum albumen	500 mg l^{-1}

2.3.9 RNA End Labelling Buffers

GES	Glycine pH9.5	5.0 mM
	(Na_2) EDTA	0.01 mM
	Spermidine	0.1 mM

MDT	Tris HCl pH7.5	500 mM
	$MgCl_2$	100 mM
	Dithiothreitol	50 mM

2.3.10 Phenol/Chloroform.

Prepared with distilled phenol as described by Maniatis *et al* (1982). Final ratios were 25 parts phenol, 24 parts chloroform, 1 part iso-amyl-alcohol and 0.1 % (w/v) 8-hydroxyquinoline.

2.3.11 Solutions for DNA Spreading by the Cytochrome C Method.

Both should by freshly prepared.

Spreading solution	60% (v/v) recrystallized formamide
	40% (v/v) 0.5 M Tris HCl pH8.5
Hypophase	20% (v/v) recrystallized formamide
	90% (v/v) 0.01 M Tris HCl pH8.5

2.4 Growth of *Rhodomicrobium vannielii*

R. vannielii was exclusively grown photoheterotrophically under anaerobic conditions in liquid PM. A variety of glass culture vessels were employed with capacites ranging from 25 ml to 20 litres. These were sealed with a tightly fitting suba seals and flushed with oxygen-free

nitrogen via needles. Duration of nitrogen flushing varied from 1 to 30 minutes depending on the vessel volume. Incubation was carried out at 30°C with an incident light intensity of 1800 lux. Culture agitation was effected by either shaking or by the use of a magnetic follower.

2.5 Maintenance of Cultures

Stocks of all culture were maintained at -20°C in 15% (w/v) glycerol/85% growth medium.

2.6 Selection of *R. vannielii* Swarmer Cells.

Selection of *R. vannielii* swarmer cells was carried out essentially as described by (Whittenbury & Dow 1977). A late exponential phase batch culture was passed down a sterile glass wool column that had previously been washed with sterile PM media. Multi-cellular complexes and stalked cells became enmeshed in the glass wool whereas swarmer cells passed through. The whole system was flushed with oxygen free nitrogen to maintain an anaerobic environment. Light was excluded by the use of tin foil to avoid premature initiation of differentiation. The quality of synchronization was monitored by either phase contrast microscopy or cell size distribution analysis.

2.7 Cell Size Distribution Analysis by Coulter Counter.

Determinations of total cell numbers and cell size were carried out on a model ZB1 Coulter Counter linked to a

Coulter Channelyzer C1000. A BBC model B microcomputer, linked directly to this system facilitated data manipulations, floppy disc storage, and printing. The software for this system was produced by M. Whiteside (unpublished data, University of Warwick).

2.8 Determination of Radioisotope Incorporation Into Cell Cultures

The method used was essentially as described by Swoboda *et al* (1982b). Triplicate 20µl samples were removed, spotted on Whatman 3mm Chr filter discs and immediately immersed into ice cold 5% (w/v) trichloroacetic acid containing 0.1% (w/v) tetra-sodium pyrophosphate. The filters were left for 20 minutes, then washed twice by replacement of this solution and twice in ice-cold ethanol. Filters were then dried and counted in 5 ml of Beckman EP Ready-solve in an LKB 1212 liquid scintillation counter. When using synchronized cells, an equal volume of unlabelled carrier cells was included to facilitate reproducable precipitation.

2.9 Protein Determinations.

The method used was essentially that of Lowry *et al* (1951) with the modifications of Herbert *et al* (1971).

2.10 Preparation of *R. vannielii* Chromosomal DNA

An equal volume of ice-cold acetone was added to a thick suspension of cells in STE in a glass universal bottle

on ice. The contents were mixed and the cells harvested by centrifugation at 4000g in a MSE Chillspin for three minutes. The resulting cell pellet was then washed repeatedly in ice-cold STE until the acetone had been removed. Final resuspension was in 10 ml of freshly prepared lysozyme solution at a concentration of 10 mg ml^{-1} in STE. 3.75 ml of 0.25 M (Na_2) EDTA pH8 was added and gently mixed in. Incubation was carried out at 37°C for 15 minutes. 2.5 mg of self-digested proteinase K in 0.5 ml was then added. 3.25 ml of 10 % (w/v)sodium dodecyl sulphate (Fisons) was then gently stirred into the cell suspension from a 5 ml pipette. Incubation at 37°C was carried out with occasional mixing until complete clearing had occurred. 4 ml of 5 M sodium perchlorate was then added followed by incubation at 60°C for 15 minutes. The lysate was then transferred to a 50 ml polypropylene Oakridge tube and was extracted twice with phenol-chloroform and twice with chloroform. 30 minutes centrifugtion at 30,000g was required to separate the phases for the first extraction. Lesser times were required for subsequent extractions.

Following extraction the nucleic acids were ethanol precipitated on ice with 2 volumes of ethanol stored at -20°C. The resulting precipitate was then spooled, transferred to a fresh Oakridge tube and dried in a vacuum desicator. Resuspension of DNA was effected in 10 ml of TE containing 100 μg ml^{-1} of heat-treated RNase. RNase digestion was carried out at 37°C for 30 minutes. The total volume was then made up to 30 ml with TE into which 30 g of caesium chloride was gently dissolved. Finally,

3 ml of 10 mg ml^{-1} ethidium bromide was added and the resulting mixture added to a 35 ml heat—sealable tube for the Beckman VTi50 rotor. Centrifugation was carried out at 165,000g for sixteen hours. The resulting DNA band was harvested. The ethidium bromide was removed by repeated isoamyl alcohol extractions and the caesium chloride by extensive dialysis against TE.

2.11 Isolation of Ribosomal Subunits from *E. coli* MRE600 and *R. vannielii*.

The method used was a modification of that described by Traub *et al* (1971).

Note: All operations were carried out on ice.
All glassware was heat-sterilized.
All solutions were treated with diethylpyrocarbonate and autoclaved.

Stage I facilitated the isolation of *E.coli* MRE600 70S ribosomes.
Stage I & Stage II were necessary for the isolation of *R. vannielii* 70S ribosomes.
Stage III allowed the separation of 50S & 30S ribosomes

Stage I

4 g of cells were washed and resuspended in 6 ml of solution I . Cell disruption was effected by two passages

through a French pressure cell (American Instrument Company) at 137 MPa. The total volume was then made up to 25 ml with solution I, transferred to an MSE 25 ml centrifuge tube and centrifuged for 30 minutes at 70,000g in an MSE 8X25ml titanium rotor. The ribosomes were harvested from the resulting supernatant by centrifugation in the same rotor at 70,000g for 6 hours. A glass homogenizer was required to facilitate resuspension of the ribosomal pellet in 2 ml of solution I. The resulting suspension was loaded onto a 30% (w/v) sucrose cushion followed by centrifugation in the same rotor for 18 hours at 70,000 g

Stage II

Resuspension of the ribosomal pellet in 1ml of Solution I was again effected using a glass homogenizer. 50 OD_{260} units aliquots of the resulting suspension was then loaded onto 34 ml, 10-25 % (w/v) (17.5 % freeze thaw) gradients in solution I in a polypropylene tubes for the Beckman SW28 rotor. Banding of the '70S' ribosomes was carried out at 80,000g for 10 hours. The gradients were fractionated using a Searle densiflow IIc attached to an ISCO Model UA-5 absorbance monitor. The lower band of three was found to contain ribosomes. The ribosomes were then pelleted in the MSE 8x25 ml titanium rotor at 130,000g for 20 hours. Stage II was repeated as necessary.

Stage III

Resuspension was on this occasion in 1 ml of solution II

which contained a low magnesium ion concentration resulting in the dissociation of ribosomal subunits. A maximum of 50 OD_{260} unit aliquots were loaded onto 10 - 25 % (w/v) gradients in solution II. These gradients were handled as above. This stage was repeated until a clear separation of peaks was obtained.

2.12 rRNA Isolation from *R. vannielii* Ribosomes

rRNA was isolated from *R. vannielii* ribosomes by repeated phenol/chloroform extractions and a subsequent ethanol precipitation at 0.1 M NaCl with 2 volumes for ethanol. Precipitation was effected by incubation overnight at $-20^{o}C$

2.13 Caesium Chloride Gradient Techniques for RNA Preparation from *R. vannielii* strain 5.

note All glassware was heat sterilised.
Where possible all solutions were treated with diethylpyrocarbonate and autoclaved.
All operations prior to the addition of caesium chloride were carried out on ice.

The cells were washed and suspended in a minimal volume of solution A. Cell lysis was effected by two passages through the French pressure cell (American instruments company) at 137 MPa. The cells were then added drop by drop to 0.04 volume of 35% (w/v) sarcosyl with continual mixing. The total volume was then adjusted to 7.2 ml into which 7.2 g of caesium chloride was dissolved. This mixture was then

loaded onto 2.7 ml cushion of solution B in a polycarbonate centrifuge tube (MSE 6x14 titanium rotor). Samples were centrifuged at 150,000g for 24 hours at 25°C. Following centrifugation the supernatant was carefully removed and the resulting RNA pellet dissolved in TE. Residual caesium chloride was removed by extensive dialysis against TE.

2.14 Large Scale Isolation of Plasmid DNA from *E. coli*.

The alkaline lysis method of Birnboim & Doly (1979) was used as described by Maniatis *et al* (1982). Volumes were reduced by 20 % to allow the use of '50 ml' Oakridge tubes. The inclusion of a centrifugation step at 30,000g for 30 minutes immediately prior to caesium chloride gradient centrifugation proved valuable in removing insoluble material from the subsequent gradient.

2.15 Rapid Small Scale Isolation of Plasmid DNA from *E. coli*.

The alkaline lysis method of Birnboim & Doly (1979) was used as described by Maniatis *et al* (1982) Washing of the DNA pellet in 70 % (v/v) ethanol was omitted as this invariably resulted in loss of plasmid.

2.16 Agarose Gel Electrophoresis of DNA.

Gel electrophoresis was carried out using tris-borate buffer (TBE).

For rapid visualization of DNA samples, a Cambridge Scientific Ltd. mini gel apparatus was used. This equipment also allowed rapid estimation of DNA concentrations (down to 500 ng ml^{-1}) by comparison with known standards.

2.17 Digesting DNA with Restriction Endonucleases.

All restriction endonucleases were used according to the suppliers (Bethesda Research Laboratories Ltd.) recommendations, though a five fold excess of enzyme was routinely employed to ensure complete digestion.

2.18 Ligation of DNA.

All reactions were carried out according to the recommendations of the suppliers of the enzyme, though a five-fold excess was routinely employed to ensure complete ligation. The relative concentrations of vector and insert DNA's used in the reaction were calculated in accordance with Maniatis *et al* (1982).

2.19 Polyacrylamide Gel Electrophoresis of DNA.

The method used was as described by Sealey & Southern (1982).

Stock Solutions	Final acrylamide concentration (%,w/v)		
	5	12	20
40% Acrylamide	12.5	30	50
2% Bisacrylamide	12.5	20	3.3
TBE 5x stock	20	20	20
TEMED	0.05	0.05	0.05
10% fresh ammonium persulphate	1.0	1.0	1.0
Distilled water	53.95	28.95	25.65

All volumes in ml.

All total volumes = 100 ml.

Gels were run at 60 volts overnight.

2.20 Agarose Gel Electrophorsis of RNA.

For the separation of rRNA's, 1.5 % (w/v) agarose gels with 50 % (v/v) formamide buffer were used. Due to the harmful nature of formamide, these gels were run in a fume hood. The following procedure is designed for a flat bed electrophoresis tank with a one litre capacity for submerged gels.

600 ml of (colourless) formamide were deionised to pH7 by stirring with 15 g of BDH Amberlite MB3 mixed bed resin. Upon reaching pH 7 vacuum aided filtration through a Buchner funnel was immediately carried out to remove the resin. One ml of this formamide was put to one side for sample

preparation. To the remainder was added 576 ml of distilled water and 24 ml of E Buffer. A portion of the resulting running buffer was used to prepare a 1.5 % (w/v) agarose slab gel (setting at 4°C). Samples were prepared by adding 1 volume of sample to 2 volumes of complete GLB. Gels were run at 40 mA constant current for about 6 hours. Staining with ethidium bromide was carried out as for a DNA gel, following a 15 minute pre-wash in distilled water to remove the formamide.

2.21 98 % Formamide/5 % Polyacrylamide Gel Electrophoresis for RNA

This method described by Grierson (1982) is reported to fully unfold RNA secondary and tertiary structure.

The gel was prepared as follows:

	10% base	5% gel
Acrylamide	2.4 g	2.4 g
Bis-acrylamide	0.1 g	0.1 g
1m NaH_2PO_4	0.25 ml	0.5 ml
TEMED	0.12 ml	0.12 ml
Fresh dionized formamide	20.0 ml	40.0 ml

These solutions were adusted to pH6 with concentrated HCl and made up to 25 ml and 50 ml respectively. Degassing was unnecessary and polymerisation was initiated by the addition of 100 µl and 200 µl respectively of freshly

prepared 36% (w/v) ammonium persulphate.

10 ml of the 10 % base was polymerized first to support the 5 % gel between the glass plates. The 5 % gel was then polymerised on top and used to form the wells. Once polymerisation was complete the gel was set up in the electrophoresis tank and the wells overlayed with neat deionized formamide.

Dried samples were resuspended in 90 % buffered formamide with 10 % (w/v) sucrose. Samples were heated to 75^{o}C for 5 minutes and loaded into the deionized formamide. Running buffer (210ml of 0.5 M NaH_2PO_4 per litre of deionized formamide adjusted to pH 6 with HCl) was carefully added to the reservoirs avoiding disturbing the samples.

The gel was run at 50 volts overnight with buffer re-cycling and using bromophenol blue as tracking dye in vacant wells. Ethidium bromide staining was effected as for DNA agarose gels following a 1 hour wash in water to remove formamide.

Note: Due to the hazardous nature for formamide all operations were carried out in a fume hood where possible.

2.22 Electroelution of DNA & RNA from Agarose Slab Gels.

Gels were stained with ethidium bromide to enable visualization of nucleic acid bands using a hand held long wave UV lamp. The gel was replaced in the gel tank and

prepared for electrophoresis using blotting paper wicks. The bands of interest were removed from the gel with a scalpel. The resulting hole was then enlarged by 1.5 cm to the front and both sides. This enlarged trough was then lined with dialysis tubing. The original gel slice, containing the band of interest, was layed in the trough. The trough was then filled with clean 0.1 x running buffer and 400 volts applied. The ethidium bromide stained nucleic acid was observed to migrate from the gel slice and concentrate against the dialysis tubing to the anode side of the trough. Reversal of the polarity of the current for 25 seconds loosened the nucleic acid from the membrane allowing sample collection in a minimum volume with a pipette. It was found to be beneficial to top up the 0.1 X running buffer in the trough and repeat the above procedure.

2.23 Southern Transfer of DNA from Agarose Gels to Nitrocellulose Paper.

The technique of Southern (1975) as described by Maniatis *et al* (1982) was used. In addition, cling film seals were placed around the perimeter of the nitrocellulose both above and below. This ensured passage of the transfer buffer through and not round this filter. 20 x SSC was used as transfer buffer and it was found that 20 minutes for both alkali denaturation and neutralization was adequate.

2.24 Colony Hybridization.

The technique of Grunstein & Hogness (1975) as described

by Maniatis *et al* (1982) was used. 170 $\mu g\ ml^{-1}$ chloramphenicol was used for plasmid amplification (in avoidance of a typographical error by Maniatis *et al* (1982).

2.25 Nick Translation of DNA

The reaction mixture was set up as follows in a 1.5 ml screw cap Eppendorf tube:

Nick translation buffer (NTB) x 10 stock	2.0 μl
DNA to be labelled 250 ng	14.5 μl
DNase I: 4×10^{-10} g ml^{-1} (freshly prepared from 1 mg ml^{-1} stock)	1.0 μl
Deoxy-nucleotide triphosphate mix (dATP, dCTP, TTP all 1 mM)	1.0 μl
^{32}P-dGTP (Amersham; specific activity 3000 Ci $mmol^{-1}$ at a concentration of 10 $\mu Ci\ \mu l^{-1}$)	1.0 μl
DNA polymerase I (BRL. 250 units per 34 μl)	0.5 μl

This solution was then mixed well and incubated at 15^{o}C for three hours. The reaction was terminated by the addition of 5 μl of 0.25 M (Na_2) EDTA pH8.

The labelled DNA was separated from the unincorporated deoxy-nucleotide triphosphates by passage through a TE-equilibrated Sephadex G50 column in a 5 ml disposable pipette.

2.26 5'-End Labelling of Ribosomal RNA

500 ng of rRNA was resuspended from ethanol precipitation in 10 μl of GES and incubated at 90°C for 30 minutes to cause limited RNA hydrolysis. The addition of 12.5 μl of MDT facilitated neutralization. 12.5 μl of ^{32}P-ATP (Amersham, specific activity 3000 Ci mmol^{-1} at a concentration of 10 μCi μl^{-1}) was added and the reaction started by the addition of 2.5 units of T4 polynucleotide kinase. Incubation was at 37°C for 30 minutes.

Unincorporated ^{32}P-ATP was removed by passage through a TE-equilibrated Sephadex G50 column in a 5 ml disposable pipette.

2.27 DNA/DNA & RNA/DNA Hybridization.

Hybridization reactions with *R. vannielii* DNA were carried out in heat-sealable bags using 6 x SSC at 79°C, or 44°C when 50 % (v/v) formamide was included. For DNA/DNA hybridization the following inclusions were made: 50 μg ml^{-1} herring testes DNA, 200 μg ml^{-1} polyvinyl pyrrolidone, 200 μg ml^{-1} ficoll and 200 μg ml^{-1} bovine serum albumen. For rRNA/DNA hybridization 50 mM Hepes pH7 and 0.5 mg ml^{-1} yeast tRNA was used, together with the constitutive use of 50 % (v/v) formamide. In either case, the probe was boiled for 15 minutes prior to addition to the above to complete the hybridization mixture. Pre-hybridizations were carried out for 3 hours and hybridizations for at least 16 hours.

The following relationships were employed in designing hybridization conditions and washing regimes for the % stringency required

1) Tm = 69.3 + 0.41 (G + C) % (Marmur & Doty 1962).

2) $(Tm_2) - (Tm_1) = 18.5 \times \log(M_2/M_1)$
where M_1 and M_2 are the respective ionic strengths of the original and new solutions. Tm_1 and Tm_2 are the respective melting temperatures for M_1 and M_2. (Dove and Davidson 1962)

3) A 1 % decrease in the number of mismatched base pairs decreases the Tm of a duplex by 1°C (Bonner *et al* 1973).

4) 1 % (v/v) formamide reduces the Tm by 0.7°C (McConaughy *et al* (1969).

Further it was taken that washing at 15°C below the Tm (for 1 x SSC) in 0.1 x SSC requires approximately 100 % homology for continued hybridization ie. 100% stringency.

When an RNA probe was used the first stage of washing was at 37°C in 2 x SSC containing heat—treated RNase A at 10 μg ml^{-1}.

At each stringency washing involved four changes of buffer at half hourly intervals.

2.28 Transformation of *E.coli* Using Calcium Chloride.

The technique used was essentially as described by Holland (1983). 1 ml of an overnight culture of *E.coli* was used to inoculate 100 ml of LB in a baffled 500 ml flask. This culture was grown to an OD_{600} of 0.5. From this point all operations were carried out on ice and using ice-cold solutions. 40 ml of culture was added to two universal bottles and left on ice to chill. Cells were pelleted by minimal spins in a multex angled contrifuge and the resulting supernatant removed by careful aspiration. Resuspension was in an equal volume of 0.1 M $MgCl_2$. Cell washing was repeated in 20 ml of 0.1 M $CaCl_2$ and the final resuspension left the cells in 2 ml of 0.1 M $CaCl_2$. These cells were held on ice for 24 hours (Dagert & Ehrlich 1979).

For transformation, plasmid DNA in 100 μl volume was mixed with 200 μl of competent cells and left on ice for 30 minutes. A two minute heat shock at 42^{o}C was applied followed by a further 15 minutes on ice. Incubation was then carried out for 1 hour at 37^{o}C following the addition of 1 ml of LB medium. After this period the transformed cells were plated onto selective media.

2.29 Induction of ppGpp and pppGpp in *E.coli* MRE600.

This was carried out as described by Mann *et al* (1975). *E.coli* MRE600 was grown in 10 ml of Davies low phosphate minimal medium using forced aeration. Once in the exponential phase 1 mCi of ^{32}P-orthophosphate was added

and the cells grown for a further 30 minutes. Trimethoprim was added to a final concentration of 50 μg ml^{-1} and incubation was continued for a further 10 minutes.

2.30 Preparation of Phosphorylated Nucleotides from *E. coli* and *R. vannielii*

0.5 ml of culture was added to 0.25 ml of 98 % (v/v) formic acid on ice and left to incubate for 20 minutes. Centrifugation was carried out in a microfuge at 4^{o}C for 10 minutes. The upper 60 % of the supernatant was taken and subjected to analysis by thin layer chromotography (TLC) and autoradiography.

2.31 Thin Layer Chromatography (TLC) of Phosphorylated Nucleotides.

Samples were spotted onto polyethyleneimine cellulose plates (20 x 20 cm), from Schleicher and Schuell. Samples were run at room temperature in a closed container using 1.5 M KH_2PO_4 pH3.6, the resulting TLC plate was dried in air and analysed by autoradiography (Mann *et al* 1975).

2.32 Determination of the S Values of Ribosomal Sub-units.

Analytical ultracentrifuation was performed in a Beckman model E analytical ultracentrifuge using a 12 mm 4^{o} KelF centrepiece in an AN-D rotor. 0.5 mg ml^{-1} samples in solution II were centrifuged at 30,000 g at 20^{o}C. Photographs were taken using ultra-violet absorption optics

at 4 minute intervals and the films examined using a Joyce-Leobel recording microdensitometer. *E. coli* MR600 ribosomal subunit standards were used. The equations described by Chervenka (1969) were used to calculate the sedimentation coefficients in Svedbergs (S).

$$S^{13} = \frac{2.303}{60w^2} \frac{d.\log x}{dt}$$

$$\text{where } w = \frac{2.\pi.rpm}{60}$$

t = time in minutes

2.33. Electron Microscopy.

Electron microscopic studies were carried out using a Joel JEM 100-S transmission electron microscope (Joel (UK) Ltd., London). Electron micrographs were taken on kodak electron image film 4463 which was developed in Kodak D19 developer and fixed in Ilford Hypam fixer.

2.33.1 DNA Spreading by the Cytochrome C Method.

Nucleoid samples were spread by the cytochrome C monolayer technique of Davis *et al* (1971). 10μl of nucleoids was carefully mixed with 50 μl of the spreading solution 40 μl distilled water and 2 μl of 0.5 % (w.v) cytochrome C, and spread on the hypophase. The film was allowed to stand for 1 minute and was picked up onto

carbon-shadowed parlodion-coated 200 mesh copper grids.

2.33.2 Negative Staining.

Samples on grids were dipped into freshly prepared 50 mM uranyl acetate in 90 % (v/v) ethanol for 20 seconds. These were then dried prior to rotary shadowing.

2.33.3 Shadowing.

Negatively stained samples on grids were shadowed at an angle of 10^{o} with platinum/palladium in a Nanotech coating unit (Nanotech (Thin films) Ltd., Prestwick, Manchester, UK).

Chapter 3

Results and Discussion

Chapter 3 Results & Discussion.

Examination of the Pattern of Synthesis of Ribosomal Components During the Cell Cycle of *R. vannielii*.

3.0 Introduction

The aim of this project was to examine the regulation of ribosome synthesis during the cell cycle of the polymorphic organism *R. vannielii*. Ribosome synthesis has only been studied in detail in *E. coli* (Nomura 1986) and to a lesser extent in *B. subtilis* (Smith 1982). Both these rod—shaped organisms have a simple vegetative life cycle involving cell elongation and subsequent division by symmetric, transverse binary fission. Under normal circumstances the process of ribosome synthesis occurs at a constant rate during the processes of cell growth and division. The rate of synthesis is only influenced by, and shows proportionality to, the growth rate (Gausing 1977). However, in addition to the vegetative cell cycle of *B. subtilis*, nutrient depletion can trigger the process of sporulation which culminates in the formation of a dormant, partially dehydrated endospore in which RNA synthesis does not occur. This process represents a diversion from the normal vegetative growth of the organism to attain an inactive state that is capable of surviving adverse environmental conditions.

In contrast, the motile swarmer cell of *R. vannielii* is a natural (though not essential) component of this organisms vegetative cell cycle. This dispersal or growth precursor

cell has analogues in a wide variety of prokaryotic organisms (Kelly & Dow 1984). However, the swarmer cell of *R. vannielii* differs from other growth precursor cells so far studied in that the initiation of maturation is blocked in the absence of an external source of energy ie. light (when grown photoheterotrophically under anaerobic conditions). In the dark the swarmer cell is unable to initiate maturation despite maintaining a high level of ATP which is not significantly altered by illumination (see figure 5.1).

The levels of ATP have been examined more closely, using a luciferin - luciferinase bio-luminescence assay during the maturation of both *R. vannielii* (Porter 1984) and *Hyphomicrobium neptunium* (Emala and Weiner 1983) swarmer cells. In both organisms the level of ATP failed to show a significant increase during the first 20 minutes following the initiation of maturation. However, it has been observed by this author that periods of illumination of less than 20 minutes duration, followed by incubation in the dark, are adequate to induce maturation (though the quality of synchrony was reduced). It appears therefore, that the swarmer cell does not have to overcome an energy threshold in the form of ATP in order to initiate maturation. Possibly a system akin to the NADP/thioredoxin system of *Chromatium vinosum* (Johnson *et al* 1984) could function in triggering swarmer cell differentiation. It should also be mentioned that when *R. vannielii* is grown chemoheterotrophically in the dark, oxygen substitutes for light in facilitating the initiation of swarmer cell maturation. This process, however, has not been studied in detail.

An important point is that once swarmer cell maturation has been initiated by light, the absolute control over that cell's destiny, afforded by darkness, is lost; i.e. when a synchronously differentiating cell population was placed in the dark, differentiation continued. It seems therefore, that the newly formed swarmer cell has a unique property in being the only *R. vannielii* cell type in which differention can be blocked by incubation in the dark.

To reiterate from chapter 1, the swarmer cell is a non-growing cell that is unable to replicate *per se*. It does not carry out DNA replication (Potts & Dow 1979) and protein synthesis is much reduced (Porter 1984),presumably to a maintenance level. The inhibited *R. vannielii* swarmer cell is not dormant however, and carries out a number of active cellular processes that are typified by vigorous motility. The inhibited swarmer cell must therefore, be under the influence of a generalized gene control system that blocks all molecular activities involved in cell growth yet at the same time allows other swarmer cell associated activities to continue. Possibly the condensed state of the swarmer cell nucleoid (Whittenbury and Dow 1977) can afford such a generalized modulation of molecular events.

As a non-growing cell with zero growth rate, the inhibited swarmer cell has no obvious requirement to synthesize new ribosomes. In such a cell, ribosome synthesis would be a very wasteful process. It was considered that the ribosomal genes of the inhibited *R. vannielii* swarmer cell

may well be subject to the same general inhibitory mechanism(s) which acts upon those genes associated with growth and maturation. In this context, the regulation *R. vannielii* ribosomal genes may differ from that observed for *E. coli* and be subject to differential cell cycle dependant control. Active synthesis of ribosomes must occur, however, either during maturation, reproduction or both in order to provide sufficient ribosomes for the next generation.

3.1 Pattern of Synthesis of rRNA During the Cell Cycle of *R. vannielii*.

3.1.1 Summary.

Pulse labelling with ^{32}P-orthophosphate showed that the synthesis of 23S and 16S rRNA occurred throughout the process of swarmer cell maturation and reproduction. The synthesis of rRNA in the dark inhibited swarmer cell could not be examined due to the cells failure to incorporate nucleotide precursors.

3.1.2 Experimental Details.

A 20 litre batch culture of *R. vannielii* was grown photoheterotrophically under anaerobic conditions on PM and swarmer cells were selected during late exponential growth. The resulting filtrate was then divided into several identical culture vessels and flushed with oxygen-free nitrogen. Incubation conditions and pulse labelling times were as detailed in figure 3.1. 2 μCi ml^{-1} ^{32}P-ortho-

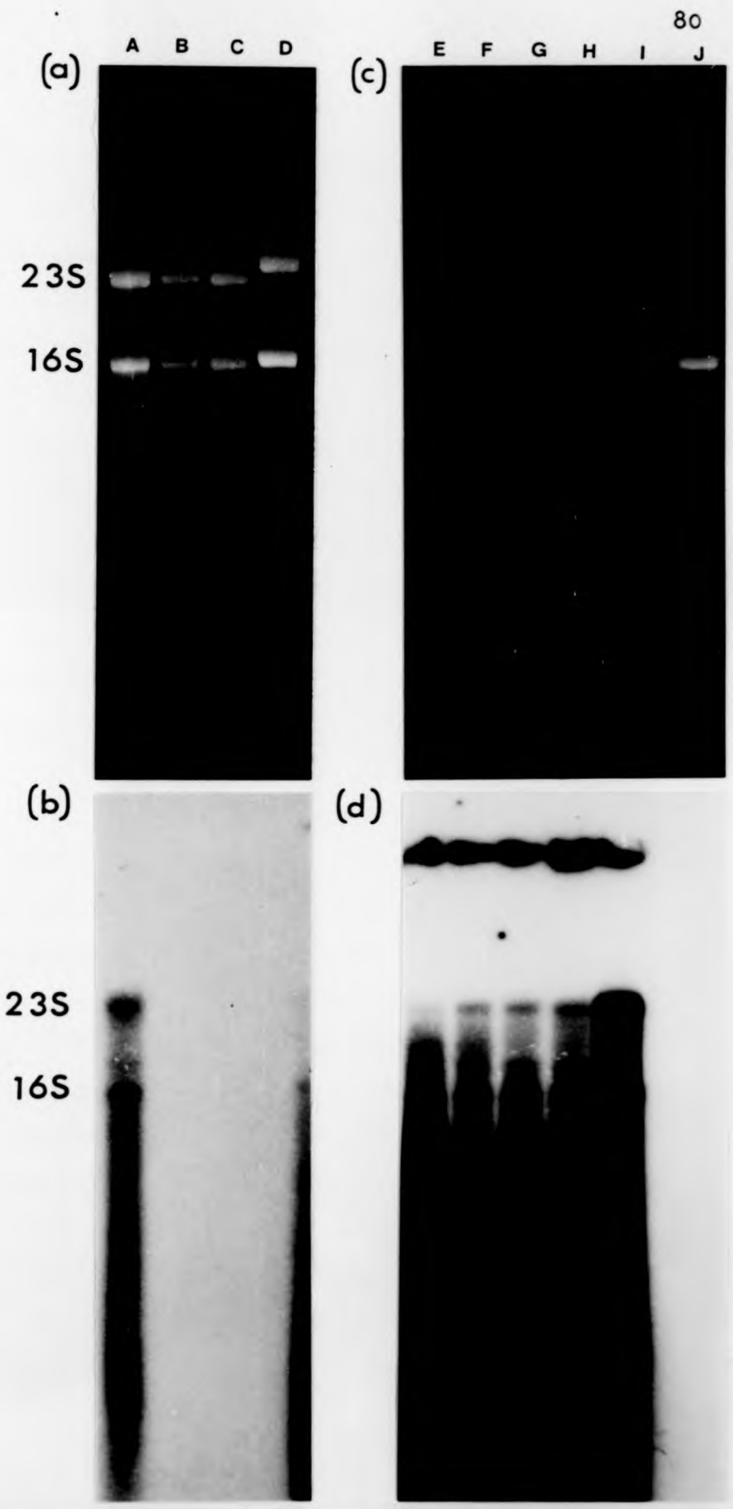
Figure 3.1.

Synthesis of Ribosomal RNA During *R. vannielii* Swarmer Cell Maturation and Reproduction as Shown by ^{32}P-orthophosphate Labelling.

R. vannielii swarmer cells were selected and either inhibited or induced to initiate maturation at time 0. Cells were pulsed with 2 μCi ml^{-1} ^{32}P-orthophosphate for the times indicated. After pulsing, total RNA was isolated and fractioned by agarose/formamide gel electrophoresis as shown by ethidium bromide staining in (a) and (c). (b) and (d) show the respective autoradiographs.

Key to tracks:

A	pulse-labelled 0 - 1 hour in the light
B	pulse-labelled 0 - 1 hour in the dark
C	pulse-labelled 0 - 2 hour in the dark
D	Unlabelled *E. coli* MRE600 rRNA control
E	Pulse-labelled 0 - 1 hour in the light
F	Pulse-labelled 1 - 2 hour in the light
G	Pulse-labelled 2 - 3 hour in the light
H	Pulse-labelled 3 - 4 hour in the light
I	Pulse-labelled 0 - 4 hour in the light
J	Unlabelled *E. coli* MRE600 rRNA control



phosphate was used for all pulse labellings which were terminated by the addition of 0.2 M phosphate buffer.

After pulse-labelling, cultures were harvested and total RNA isolated by the caesium chloride gradient method. The resulting RNA was fractionated by agarose/formamide gel electrophoresis and visualized by ethidium bromide staining. These gels were then dried and subjected to autoradiography.

The results in figure 3.1 show that the synthesis of *R. vannielii* 23S and 16S rRNA occurs throughout a four hour period of swarmer cell maturation and reproduction. Labelling of RNA species smaller than the 16S rRNA i.e. putative mRNA, indicates a very rapid rate of synthesis throughout this same period.

It was not possible to obtain direct evidence on the level of rRNA synthesis in the inhibited swarmer cells by this method as they were shown not to incorporate ^{32}P-orthophosphate (figure 3.2). It was also found that inhibited swarmer cells failed to incorporate ^{3}H-uridine, ^{3}H-adenosine, ^{32}P-adenosine triphosphate, ^{3}H-uracil or ^{3}H-cytosine. In addition, Potts and Dow (1979) have reported very poor incorporation of ^{3}H-thymidine into *R. vannielii* in general. The inability of dark-inhibited *R. vannielii* swarmer cells (grown photoheterotrophically under anaerobic conditions) to incorporate nucleic acid precursors is typified by the results shown in figure 3.2 showing ^{32}P-orthophosphate labelling where incorporation by

Figure 3.2.

Uptake of ^{32}P-orthophosphate by R. vannielii Swarmer Cells Under Various Regimes of Illumination.

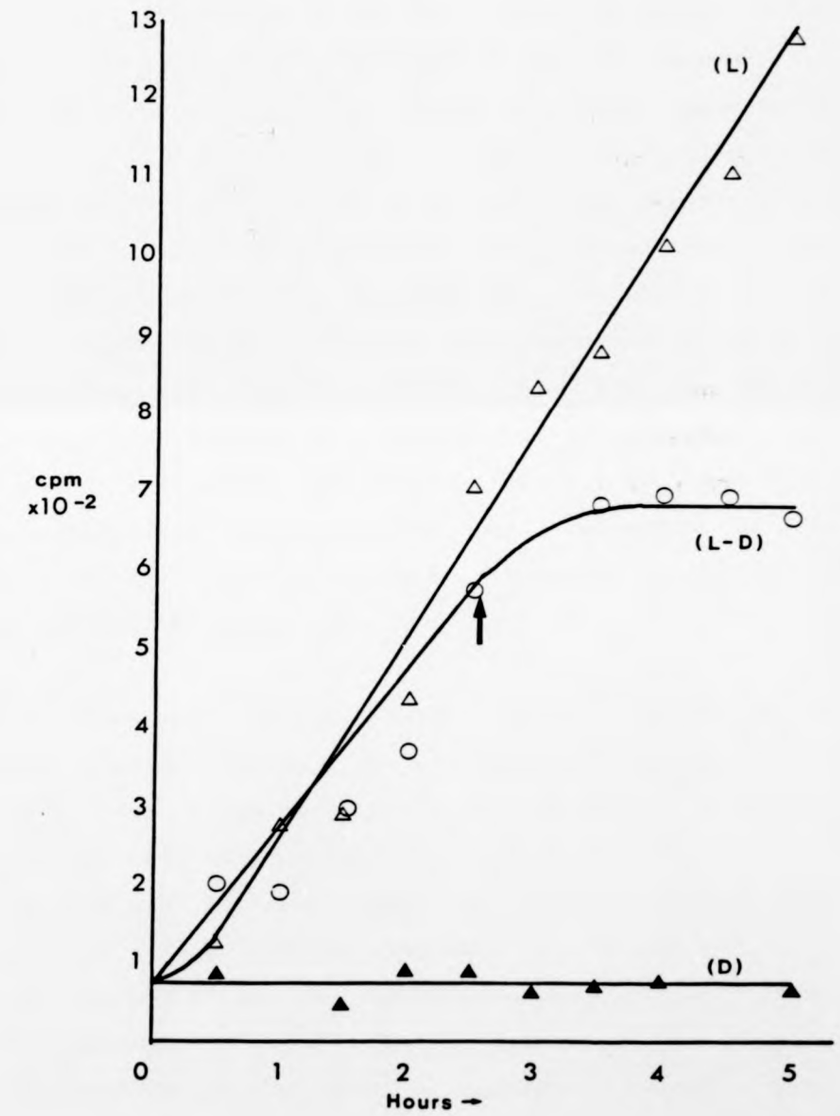
R. vannielii swarmer cells were selected and divided into three 100 ml cultures. ^{32}P-orthophosphate was added to each culture to give a concentration of 1 μCi ml^{-1}. Growth conditions and sampling times were as indicated:

Key:

(L) Incubation in the light

(D) Incubation in the dark ie. inhibition of swarmer cell development.

(L-D) Incubation in the light for 2.5 hours followed by incubation in the dark (ie early stalked cells)



differentiating swarmer cells in the light is clear. An apparent low and variable level of incorporation was found to occur during some determinations, but this was found to be related to the quality of synchronization. No incorporation was observed when dark incubated swarmer cells showed no evidence of differentiation over a several hour period, as determined by cell volume analysis (figure 3.3). In addition, ^{32}P-orthophosphate incorporation was also terminated in differentiating cells that were placed in the dark (figure 3.2). This observation suggests that the apparent ^{32}P-orthophosphate incorporation by swarmer cells in the dark results from a switch from photoheterotrophic to chemoheterotrophic growth rather than from a limited initiation of maturation caused by inadequate light exclusion. Such a switch may easily result from inadequate anaerobic technique during swarmer cell selection or the admission of air into cultures by repeated sampling with needles through the suba seals.

There are several possibilities to explain the failure of inhibited swarmer cells to incorporate nucleic acid precursors. The first is to argue that an energy conserving cell that is only turning over mRNA and not synthesising new rRNA or DNA has no requirement to import nucleic acid precursors. A second possibility may be an inhibition of membrane transport in the inhibited swarmer cell. Reduced membrane permeability in the inhibited swarmer cell has also been shown for rifampicin (Scott & Dow, SGM in press) and the tetraphenylphosphonium cation (TPP^+) (Kelly 1986). However, the incorporation of ^{35}S-methionine was shown to

Figure 3.3.

Cell Size Distribution Analysis of *R. vannielii* Swarmer Cells at Various Stages of Differentiation.

R. vannielii swarmer cells were selected and subjected to cell size distribution analysis at the times indicated:

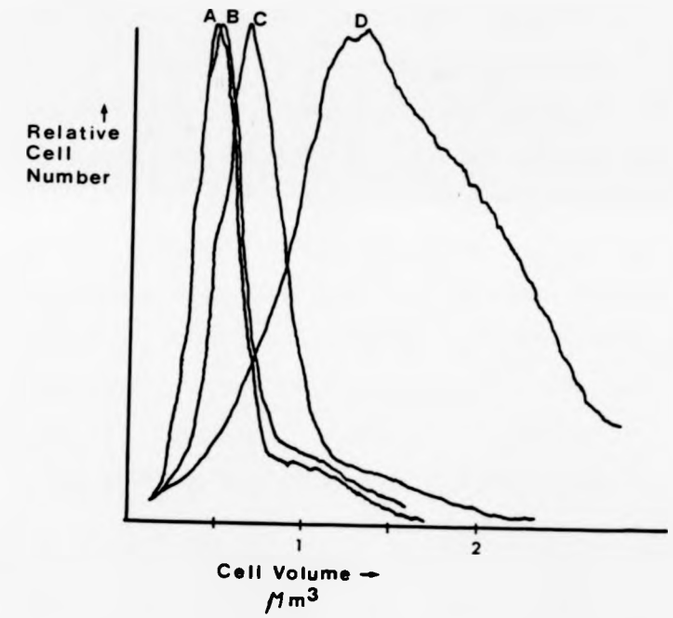
Key:

A Selected swarmer cells at time 0

C Non-motile cells following 2 hours incubation in the light.

D Cell pairs following 4 hours incubation in the light.

B Inhibited swarmer cells after 4 hours incubation in the dark



occur in inhibited swarmer cells (figure 3.5), albeit at a reduced rate. The third possibility is that membrane transport is inhibited in the dark.

3.2 Development of a Method for *In Vitro* Transcriptional Run-Off From *R. vannielii* Nucleoids.

3.2.1 Aims.

The aim of this section was to examine *in vitro* transcription from isolated *R. vannielii* nucleoids as a means by which to study the regulation of rRNA synthesis. Such a transcription system would provide a way of circumventing possible variations in membrane permeability. rRNA transcription products may be detected by hybridization to rDNA clones (chapter 4). This study required developing a reliable method by which to isolate functionally active membrane associated nucleoids from *R. vannielii* and couple this to an *in vitro* system to facilitate the completion of transcripts that had been initiated *in vivo*.

3.2.2 Summary.

A method for isolating membrane associated nucleoids from *R. vannielii* was developed. A subsequent *in vitro* transcription assay was employed and showed incorporation of ^{3}H-uridine into TCA-precipitable material. This assay was not reproducible.

3.2.3 Experimental Details.

A modification (Swoboda *et al* 1982a) of the method of Stonington and Pettijohn (1971) to isolate membrane associated nucleoids from *C. cresentus* CB15 was initially tried. Due to the instability of nucleoid preparations Stonington and Pettijohn stressed the importance of both speed and low temperature ($0^{o}C$) for reproducible results. Their method for cell lysis takes only several minutes with all steps being carried out on ice. In essence this method involves a thirty second lysozyme treatment and is completed by the addition of the non-ionic detergents Brij 58 and sodium deoxycholate (1 % and 0.4 % w/v respectively). Most published methods for other organisms represent minor modifications of this procedure (eg Swoboda *et al* 1982a). However, due to the refractory nature of *R. vannielii* to cell lysis, this procedure and its many variations, were found to be inadequate. The following procedure was finally adopted for its reliability in causing cell lysis and the subsequent production of cell associated nucleoids, as confirmed by electron microscopy (figure 3.4.b).

All steps were carried out on ice unless otherwise stated. 10^{10} cells were harvested and resuspended in 600 μl of solution A (0.01 M Tris HCl pH8.2, 0.1 M NaCl, 20 % (w/v) sucrose) in an 1.5 ml microfuge tube. Following the addition of an equal volume of acetone, this suspension was vortexed for 3 seconds and the cells immediately harvested by centrifugation (50 seconds). Repeated washing with solution

A was effected to remove all traces of acetone (approximatey 6 washes). The acetone wash was performed to remove lipopolysacharride from the outer surface of the cell which would otherwise interfere with the action of lysozyme. The treatment was found to reduce the viability of the cells by 6 logs.

The final resuspension was in 1 ml of 6 mg ml^{-1} (freshly prepared) lysozyme solution in 0.12 M Tris HCl pH8.2, 0.05 M (Na_2) EDTA. Incubation was carried out at 20°C for 5 minutes. Final lysis was achieved by adding 5 ml of 1 % (w/v) sodium dodecyl sulphate, 0.1 M (Na_2) EDTA, 0.01 M spermidine hydrochloride pH 8.2.

Nucleoids were immediately purified by loading 2.5 ml of lysate onto an 8.5ml, 10 to 30 % (w/v) sucrose gradient in a polycarbonate centrifuge tube for the MSE 6 x 14 ml titanium swing-out rotor. The sucrose gradients were prepared in 0.01 M Tris HCl pH 8.2, 1 mM (Na_2) EDTA, 5mM $MgCl_2$, 1 mM 2-mercaptoethanol. Centrifugation was carried out at 25,000 g for 5 minutes. The resulting gradients were then fractionated using a Searle densiflow IIc attached to an Isco model UA-5 absorbance monitor. A typical profile is shown in figure 3.4.a. Heterogeneous cell populations were found to cause a problem in cases of incomplete lysis as unlysed cells co-banded with lysed cells due to the inter-connecting prosthecae.

In vitro transcription was carried out as described by Swoboda *et al* (1982b). Reaction mixtures were set up as

Figure 3.4.

Figure 3.4(a).

Sucrose Gradient Profile of Nucleoids from *R. vannielii* Early Stalked Cells.

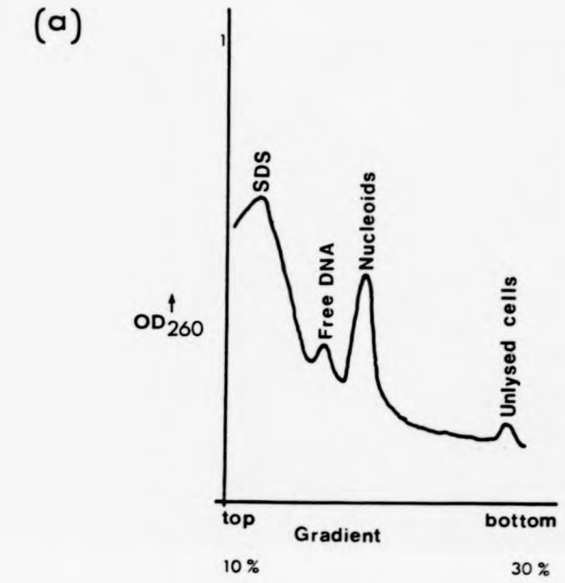
R. vannielii early stalked cells (2 hours) were lysed as described in the text and loaded onto a sucrose gradient to facilitate separation of the cell associated nucleoids from both free DNA and unlysed cells.

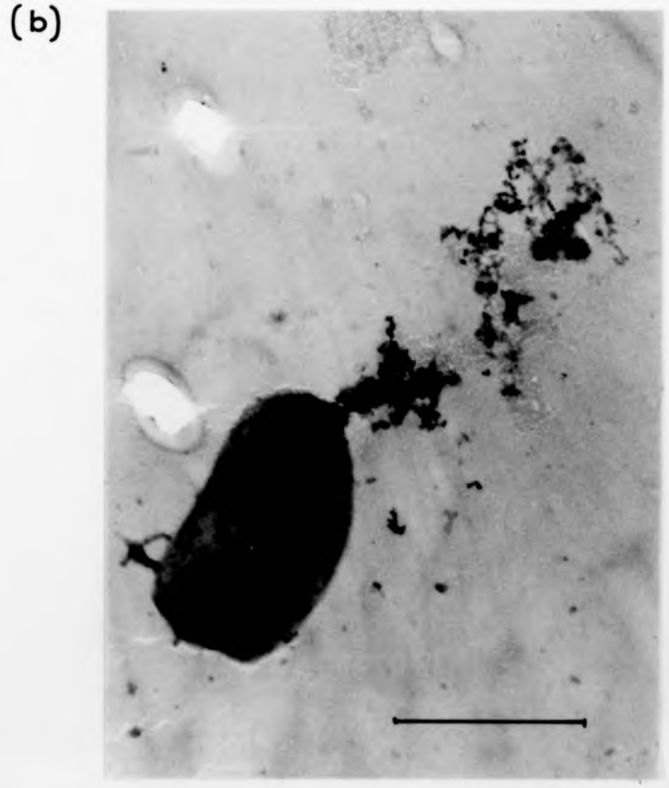
Figure 3.4(b).

Electron Micrograph of an Envelope Associated Nucleoid From an *R. vannielii* Pre-Stalked Cell.

Nucleoids were prepared and spread for electron microscopy as detailed in chapter 2.

Bar represents 1/*m*.





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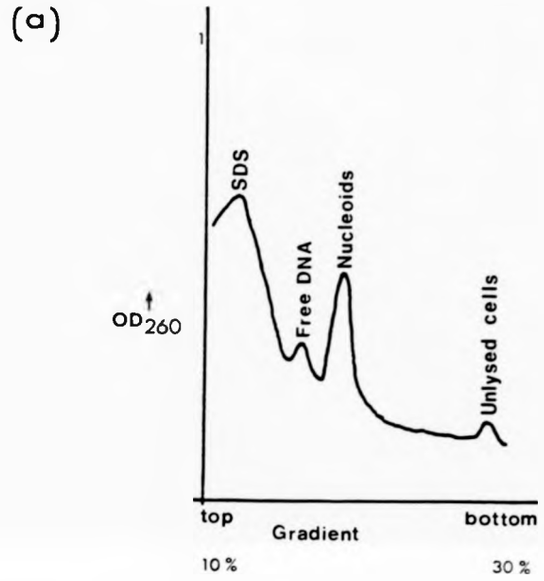


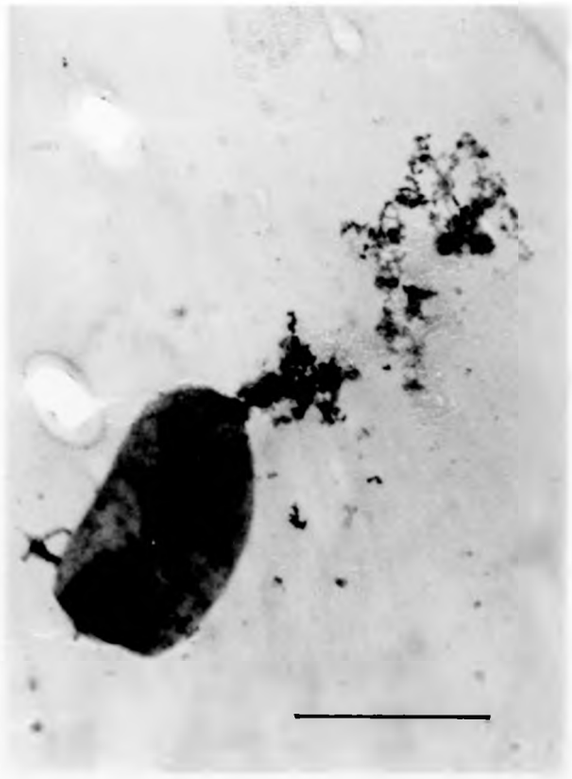
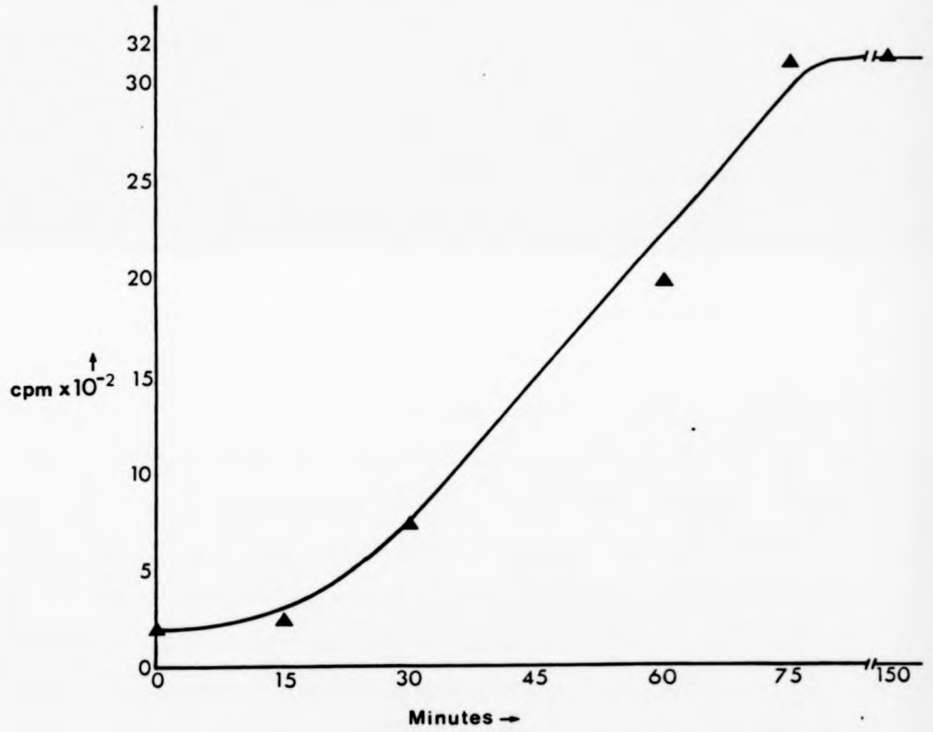


Figure 3.5.

Incorporation of ^{3}H-uridine triphosphate into TCA-Precipitatible Material by Nucleoids Isolated from *R. vannielii* Early-Stalked Cells in an *In Vitro* Transcription System.

Cell associated nucleoids were isolated as described in the text and used in an *in vitro* transcription system. The figure shows the best incorporation of ^{3}H-uridine achieved in such an assay.



follows in a total volume of 200 µl: 1 mM each of ATP, CTP, GTP; 0.05 mM UTP, 1 µCi of ^{3}H-UTP (Specific activity, >400 Ci mmol^{-1} at a concentration of 10 mCi ml^{-1}); 12.5 mM MgCl 100 mM Tris HCl pH 7.5; 7.5 mM 2-mercaptoethanol; 1 mM spermidine hydrochloride; 0.4 OD_{260} units of nucleoids. Reactions were started by transferring the solutions from ice to 37°C. 20 µl samples were taken at 15 minute intervals and prepared for determination of radioisotope incorporation as described in Chapter 2.

Figure 3.5 shows the results achieved by this method for differentiating early stalked cells. Unfortunately, this method did not reliably show an increase in the incorporation of (^{3}H)-UTP into TCA-precipitable material dispite numerous attempts to standardize the procedure.

It was concluded that the stage most likely to produce variability was that of cell lysis, as the method described relied on an acetone pre-wash, the ionic detergent sodium dodecyl sulphate and elevated temperatures. Extensive efforts were made to reduce all potentially problematic parameters, but the method described above represented a minimum treatment in this researcher's hands by which to achieve reproducable gentle cell lysis of this characteristically resilient organism (The degree of lysis was dramatically reduced by lowering the proportion of acetone in the pre-wash, or the duration or temperature of the lysosyme treatment. Raising the concentration of the lysosyme produced no beneficial effect. Adequate cell lysis could not be achieved using the detergents Brij 58, sodium deoxycholate

or Triton X100. 1 % sarcosyl effected reproducible lysis,but no incorporation of ^{3}H-UTP into TCA-precipitable material was achieved using this detergent. Butanol can substitute for acetone to obtain cell lysis but is difficult to wash free from the treated cell's and no incorporation of ^{3}H-UTP was achieved using this solvent). This line of approach was taken no further, but provides a firm basis from which to perfect the methodology.

3.3. Examination of the Pattern of r-Protein Synthesis During the Cell Cycle of *R. vannielii*.

3.3.1 Summary.

^{35}S-methionine was shown to be incorporated by dark - inhibited *R. vannielii* swarmer cells and a method for the isolation of *R. vannielii* ribosomes was developed. Pulse - labelling with ^{35}S-methionine indicated that r-protein synthesis does occur in the dark-inhibited swarmer cell.

3.3.2 Uptake of ^{35}S-methionine by Both Differentiating and Inhibited Swarmer Cells of *R. vannielii*.

A 5 litre batch culture of *R. vannielii* was grown photoheterotrophically on PM medium and the swarmer cells selected during late exponential growth. These cells were then divided into several identical 100 ml cultures and flushed with oxygen-free nitrogen. Incubation conditions and pulse labelling times were as detailed in figure 3.6.

Figure 3.6.

Uptake of ^{35}S-methionine by R. vannielii Swarmer Cells Under Various Regimes of Illumination.

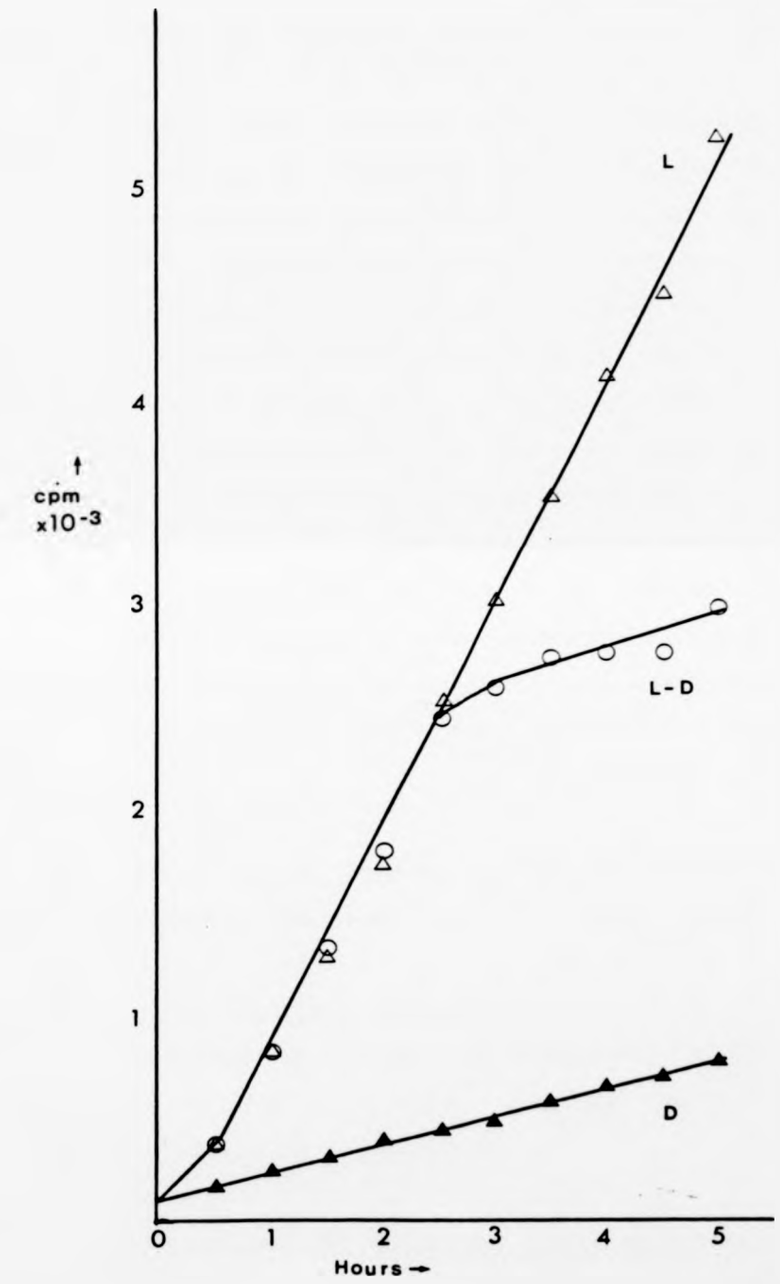
R. vannielii swarmer cells were selected and divided into three 100 ml cultures. ^{35}S-methionine was added to each culture to a concentration of 2 μCi ml^{-1} with 2.5 μM unlabelled methionine. Incubation and sampling times were as indicated:

Key:

(L) Incubation in the light

(D) Incubation in the dark ie. inhibition of swarmer cell differentiation.

(L-D) Incubation in the light for 2.5 hours followed by incubation in the dark.



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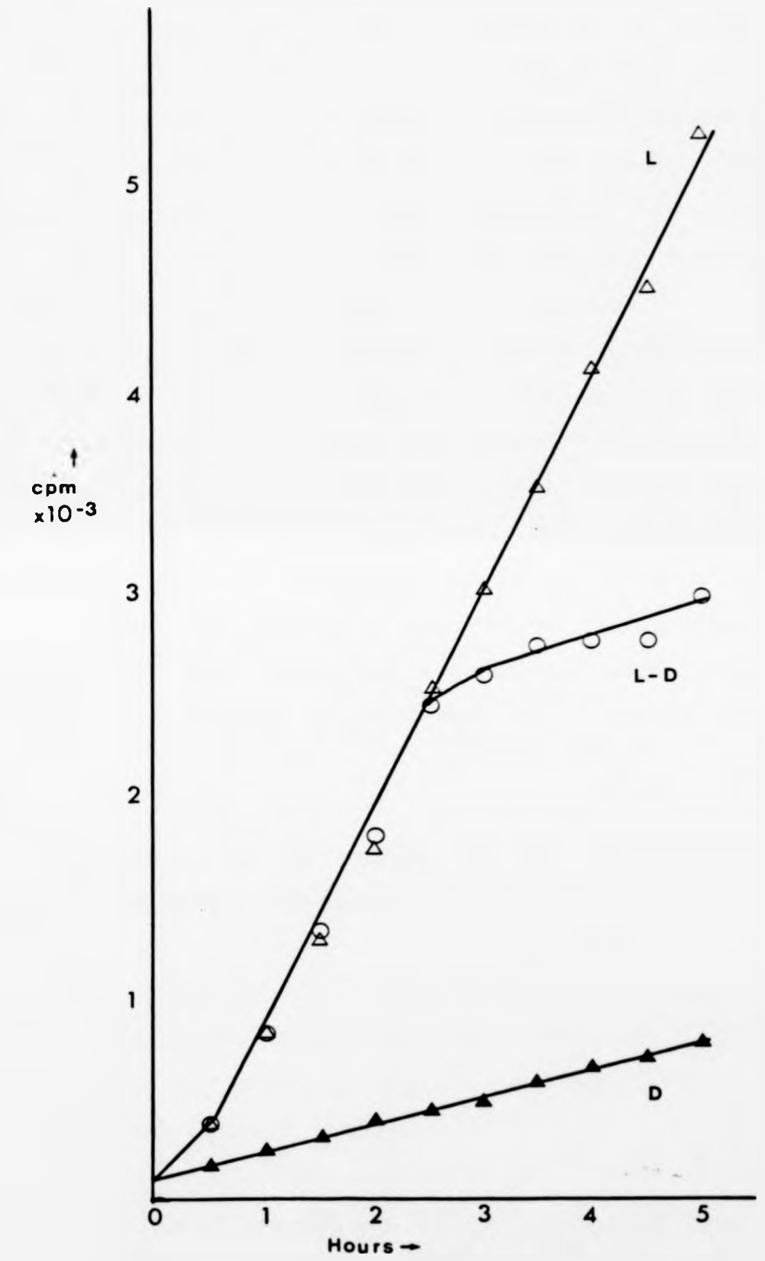


2 μCi ml^{-1} ^{35}S-methionine was used for labelling with 2.5 μM unlabelled methionine. No differentiation of swarmer cells occurred when cultures were incubated in the dark.

It can be seen from figure 3.6 that ^{35}S-methionine incorporation does occur in dark-inhibited swarmer cells. The rate of incorporation by inhibited swarmer cells in the dark was found to be 12.8 % of that seen for differentiating swarmers in the light. This reduction may well reflect a reduction in the rate of protein synthes in the inhibited swarmer cell, ie. this cell is turning over protein, not synthesizing additional protein and therefore utilizes less external amino acid. A reduction in the rate of incorporation of ^{35}S-methionine is also observed in differentiating prosthecate cells when placed in the dark, though these cells continued the process of maturation and reproduction. This observation was taken to suggest that under anaerobic conditions, light may be required for efficient methionine uptake.

3.3.3 Development of a Method for the Purification of *R. vannielii* Ribosomal Subunits.

The buffers described by Traub *et al* (1971) were used, though other details of this method were modified. Conditions were optimized for the isolation of ribosomal subunits from *E. coli* MRE600 as shown in figure 3.7.

Essentially, the method used for the purification of ribosomal subunits from *E. coli* involved the following

(see chapter 2) : physical cell lysis; removal of coarse cell debris by slow centrifugation; harvesting of 70S ribosomes by high speed centrifugation; purification of 70S ribosomes by pelleting through a 30 % (w/v) sucrose cushion; separation of the ribosomal subunits by sucrose gradient centrifugation at a low magnesium ion concentration (a low magnesium ion concentration facilitates dissociation of the 70S ribosomes into its 30S and 50S components).

The *E. coli* optimized method was then applied to *R. vannielii* resulting in the low magnesium sucrose gradient profile of figure 3.8.a. This atypical profile was reproducible and taken to indicate that contaminants were co-banding with the 30S ribosomal subunit of *R. vannielii*. Electron microscopy confirmed that the 70S ribosomes of *R. vannielii* were contaminated at this stage (figure 3.8.b). Stage II of the *R. vannielii* ribosome purification method was introduced to remove those contaminants. This involved separating the intact 70S ribosome from the lower S-value contaminants by banding on a high magnesium sucrose gradient as shown in figure 3.9.a. Three peaks were obtained and the lower band was confirmed to contain 70S ribosomes by the presence of both 16S and 23S rRNA. The subsequent separation of ribosomal subunits by low magnesium sucrose gradient centrifugation showed 30S and 50S peaks in the correct stoichiometric relationship (figure 3.9.b).

Figure 3.7.

Optimization of the Isolation of *E. coli* MRE600 Ribosomal Subunits on Sucrose Gradients.

Ribosomes were prepared as described in materials and methods and the subunits separated on 10 - 25 % (w/v) low magnesium sucrose gradients. Samples loadings were as indicated:

Key:

(a) 5 OD_{260} units loaded
(b) 20 OD_{260} units loaded
(c) 50 OD_{260} units loaded
(d) 150 OD_{260} units loaded

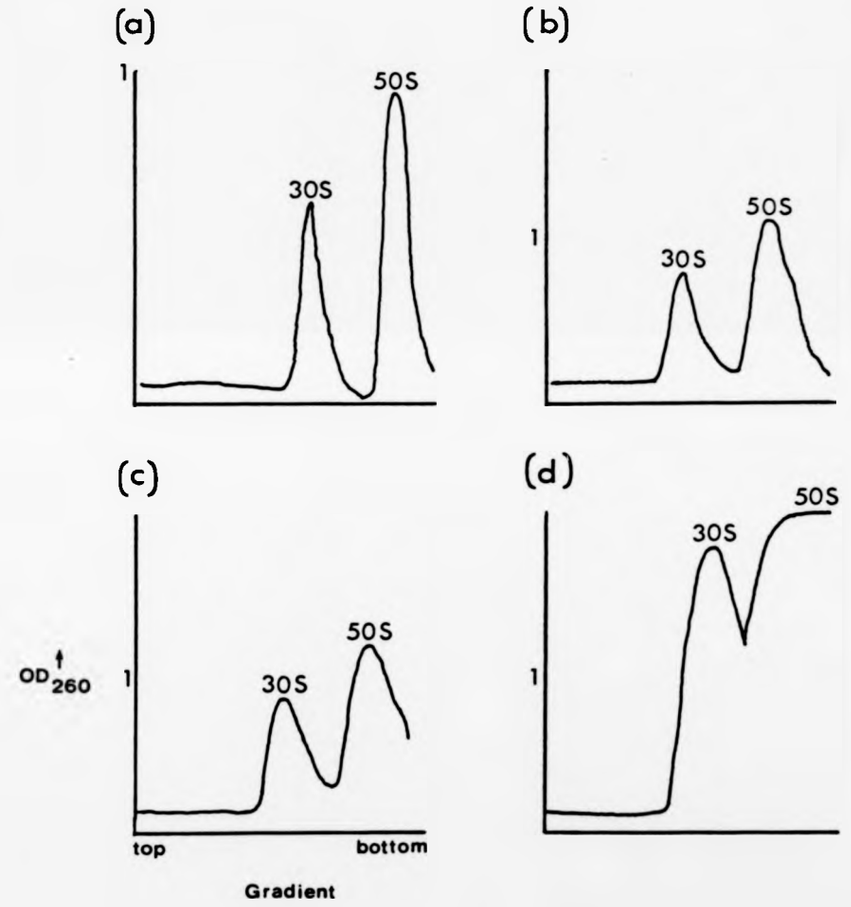


Figure 3.8.

Figure 3.8.a.

Purification of *R. vannielii* Ribosomal Subunits by Sucrose Gradient Centrifugation.

R. vannielii ribosomes were isolated as described in Chapter 2, omitting stage II. The subunits were separated on 10 - 25 % (w/v) low magnesium sucrose gradients. This profile (cf. figure 3.6) suggests co-banding of a contaminant(s) with the 30S subunit.

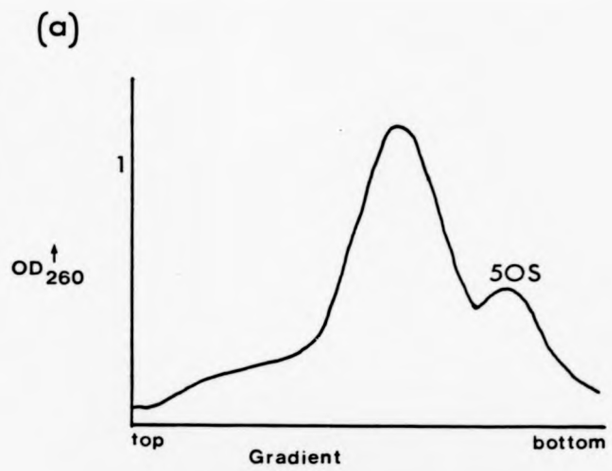


Figure 3.8.b.

Electron Micrograph of *R. vannielii* Ribosomes During Purification.

R. vannielii 70S ribosomes were prepared as described in Chapter 2, stage I.

Bar represents 0.5 μm.

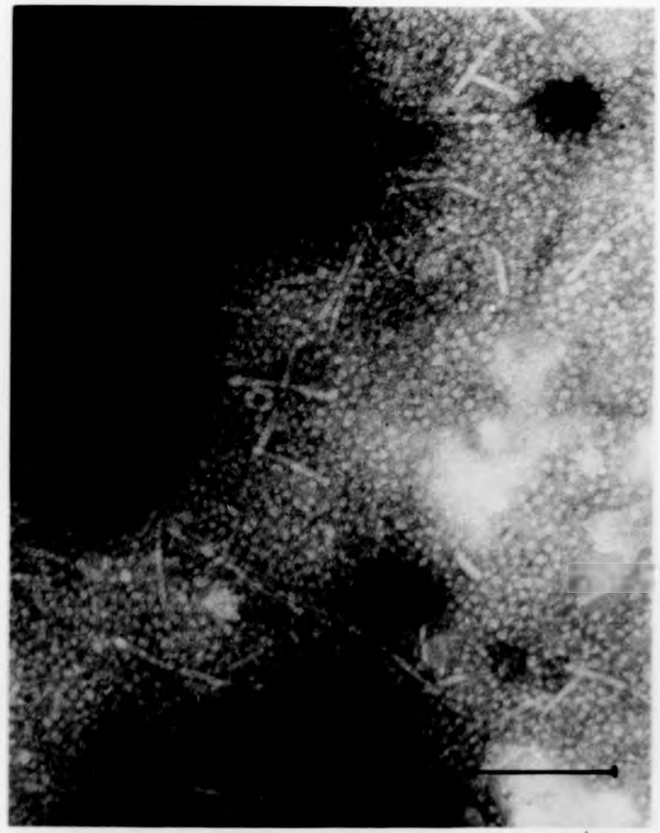


Figure 3.8.

Figure 3.8.a.

Purification of *R. vannielii* Ribosomal Subunits by Sucrose Gradient Centrifugation.

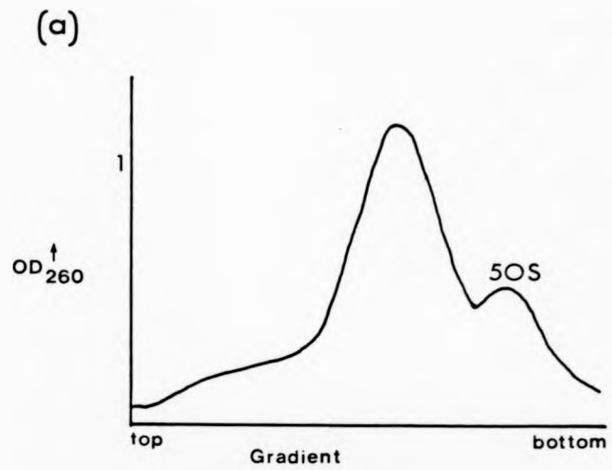
R. vannielii ribosomes were isolated as described in Chapter 2, omitting stage II. The subunits were separated on 10 - 25 % (w/v) low magnesium sucrose gradients. This profile (cf. figure 3.6) suggests co-banding of a contaminant(s) with the 30S subunit.

Figure 3.8.b.

Electron Micrograph of *R. vannielii* Ribosomes During Purification.

R. vannielii 70S ribosomes were prepared as described in Chapter 2, stage I.

Bar represents 0.5 /m.



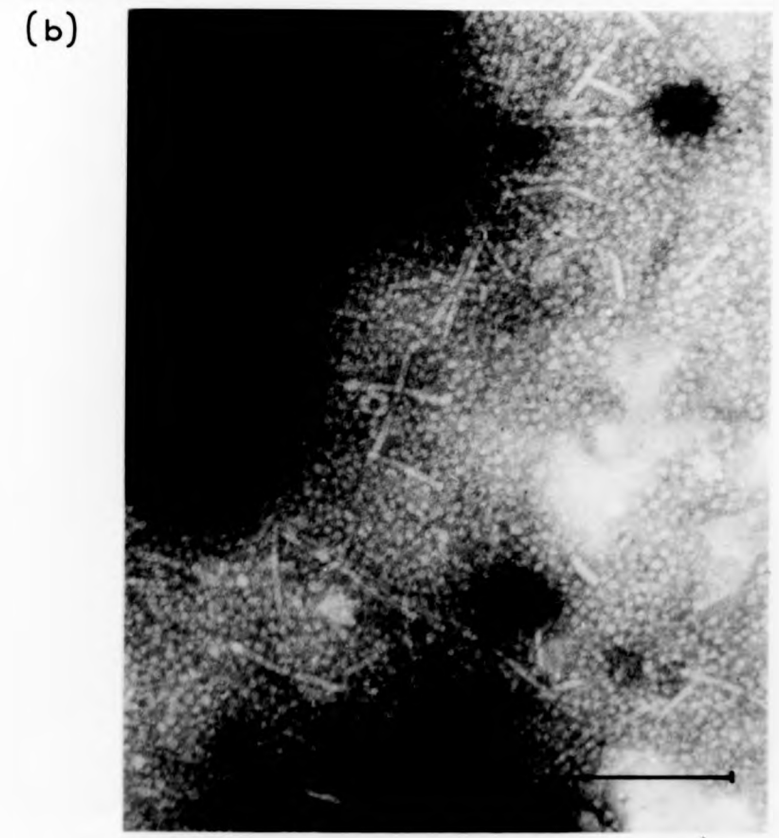


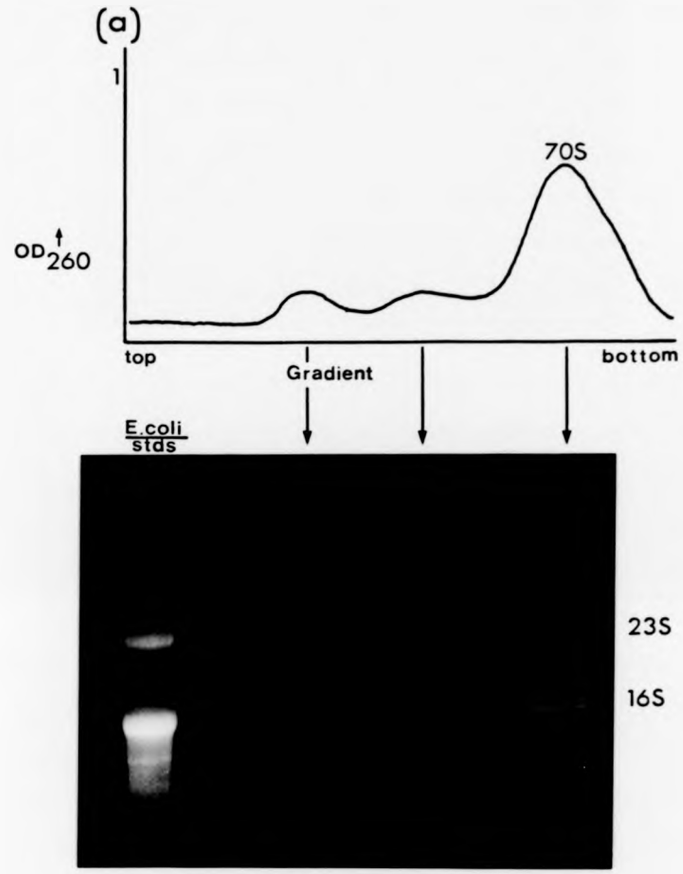
Figure 3.9.

Purification of *R. vannielii* Ribosomal Subunits by Sucrose Gradient Centrifugation.

R. vannielii ribosomes were isolated as described in chapter 2.

Figure 3.9.a. shows the OD_{260} profile during 70S ribosome purification on a high magnesium sucrose gradient in stage II. The ethidium bromide stained gel shows the position of the 70S ribosomes within the lower peak by the presence of rRNA.

Figure 3.9.b. shows the separation of the ribosomal subunits from the purified *R. vannielii* ribosome on a low magnesium sucrose gradient.



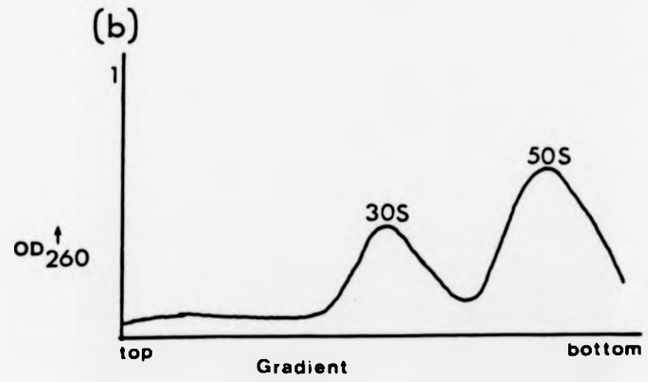


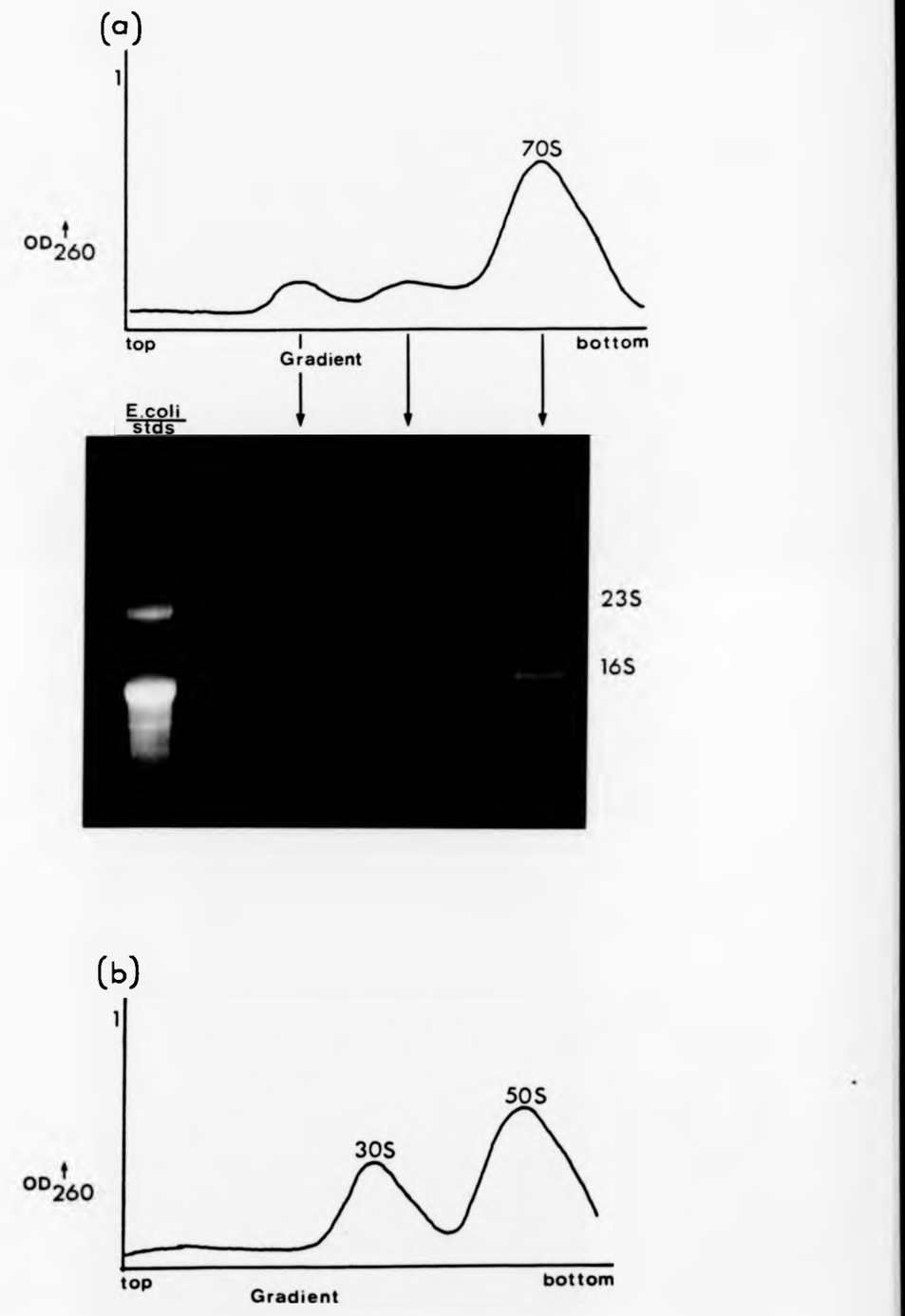
Figure 3.9.

Purification of *R. vannielii* Ribosomal Subunits by Sucrose Gradient Centrifugation.

R. vannielii ribosomes were isolated as described in chapter 2.

Figure 3.9.a. shows the OD_{260} profile during 70S ribosome purification on a high magnesium sucrose gradient in stage II. The ethidium bromide stained gel shows the position of the 70S ribosomes within the lower peak by the presence of rRNA.

Figure 3.9.b. shows the separation of the ribosomal subunits from the purified *R. vannielii* ribosome on a low magnesium sucrose gradient.



3.3.4 Comparison Between the Rates of r-Protein Synthesis in Inhibited and Differentiating *R. vannielii* Swarmer Cells .

Selected swarmer cells were divided into three identical 1 l cultures. ^{35}S-methionine was added to a concentration of 2 μCi ml^{-1} with 2.5 μM unlabelled methionine. Labelling was carried out for either one hour in the dark or one hour in the light. Pulsing in the control culture was terminated immediately following the addition of ^{35}S-methionine by the further addition of 200 mM cold methionine. 10^6 cells were taken for determination of ^{35}S-methionine incorporation at the beginning and the end of the pulse time. 70S ribosomes were isolated from the remaining cells as described in Chapter 2 with the inclusion of cold carrier cells to facilitate handling. The level of ^{35}S-methionine incorporation into the 70S ribosomes was also determined. The results are summarized in table 3.1.

The first observation that can be made from examining the results is the relatively high level of incorporation in the negative controls. This may be explained if the initial pulse of ^{35}S-methionine had been rapidly taken up by the cells to produce an intracellular pool of ^{35}S-methionine. In such a situation the subsequent chase with unlabelled methionine would be unable to displace this pool. Previous labelling experiments by this author have indicated that 20 mM unlabelled methionine (one tenth of the chase used here) abruptly terminated ^{35}S-methionine incorporation. However, it was considered that the inclusion of these negative controls could lead to a misleading interpretation of the

Table 3.1.

Comparison Between the Rates of ^{35}S-methionine Incorporation Into the Ribosomes of *R. vannielii* Swarmer Cells That Are and Are Not Undergoing Maturation.

	cpm in 10^6 cells	cpm in 0.2 OD_{260} units of 70S ribosomes
Control	103	97
1 hr in dark	543	165
1 hr in light	3304	1399

R. vannielii swarmer cells were selected and divided into three 1 litre cultures. ^{35}S-methionine was added to a concentration of 2 μCi ml^{-1} with 2.5 μM unlabelled methionine. Labelling was carried out for either 1 hour in the dark or in the light as indicated. Pulse labelling in the control was terminated immediately after addition of the label by the addition of unlabelled methionine (to 200mM). 10^6 swarmer cells were taken for determination of ^{35}S-methionine incorporation at the end of the pulse. 70S ribosomes were prepared as described in Chapter 2 with the inclusion of cold carrier cells to facilitate handling.

results and shall therefore be disregarded.

The rate of incorporation of ^{35}S-methionine into inhibited and maturing *R. vannielii* swarmer cells coincides fairly well in terms of relative efficiencies with that shown in figure 3.6. The previous determination showed inhibited swarmer cells to incorporate ^{35}S-methionine at 12.8 % the rate shown by differentiating swarmer cells compared to the figure of 16.4 % (543/3304 x 100 %) found here. Again it was considered likely that these figures reflect a reduction in the capacity of the non-growing inhibited swarmer cell to utilize methionine, although the reduction could reflect a reduced membrane permeability with respect to ^{35}S-methionine.

If the ^{35}S-methionine requirement of the inhibited swarmer cell was low, the results suggest that the rate of ^{35}S-methionine incorporation into ribosomes in the inhibited swarmer cell is ten fold lower (11.8 % i.e. 165/1399 x100 %) than that found in its maturing counterpart. Alternatively, if membrane permeability is a limiting factor in the inhibited swarmer cell, consideration must be taken of the fact that these cells only have 'access to' 16.4 % of the available ^{35}S-methionine. In this latter case the results would imply a very small reduction (11.8/16.4 x 100 % = 72 %) in the rate of ^{35}S-methionine incorporation into ribosomes of the dark inhibited swarmer cell, in comparison with its maturing counterpart. However, it seems unlikely that a non-growing cell would maintain the unnecessary and therefore wasteful process of ribosome synthesis to almost as high a level as that found in an actively growing cell.

Finally, irrespective of the role of membrane permiability, it is clear that in the dark inhibited swarmer cell, r-protein is being synthesized and incorporated into ribosomes, despite the absence of cell growth. These results (though unclear in quantitation) therefore imply that ribosomes are being turned over in the dark inhibited swarmer cell

It should be noted that the methodology used was not a direct measure of the rate of r-protein synthesis. This approach would not have detected the synthesis of r-proteins that were not incorporated into ribosomes and could not show ribosome turnover.

3.4 Characterization of the *R. vannielii* Ribosome.

3.4.1 Summary.

The two major rRNA species of *R. vannielii* were found to be similar in size to the 23S and 16S rRNA's of *E. coli*. On the other hand, the 46S and 26S ribosomal subunits of *R. vannielii* were both found to be smaller than their respective 50S and 30S *E. coli* counterparts.

3.4.1 Determination of the Size of *R. vannielii* rRNA

This examination was initiated by the observation that the *R. vannielii* 23S rRNA appeared significantly smaller than the *E. coli* 23S rRNA as seen on agarose/formamide gels (see figure 3.1.a). Comparisons were carried out using a fully

Figure 3.10.

Characterisation of *R. vannielii* rRNA.

Comparison between the sizes of the two major rRNA's of *R. vannielii* with the 23S and 16S rRNA's of *E. coli* MRE600 and the 28S and 18S rRNA's of wheat germ on a fully denaturing 98 % (w/v) formamide gel.

Key

A	*R. vannielii* rRNA
B	Wheat germ rRNA
C	*E. coli* MRE600 rRNA

Figure 3.10.

Characterisation of *R. vannielii* rRNA.

Comparison between the sizes of the two major rRNA's of *R. vannielii* with the 23S and 16S rRNA's of *E. coli* MRE600 and the 28S and 18S rRNA's of wheat germ on a fully denaturing 98 % (w/v) formamide gel.

Key

A	*R. vannielii* rRNA
B	Wheat germ rRNA
C	*E. coli* MRE600 rRNA

denaturing 98 % (v/v) formamide/5 % (w/v) polyacrylamide gel system. Wheat germ 28S and 18S rRNA's (a gift from Dr. A. Cuming) were also included for comparison. It was concluded that *R. vannielii* and *E. coli* rRNA's were comparable in size and that previous apparent differences resulted purely from the effect of secondary and tertiary RNA structure (figure 3.10).

3.4.3 Determination of the Size of *R. vannielii* Ribosomal Subunits.

Having developed a method of purifying *R. vannielii* ribosomes it was then possible to size the individual subunits by analytical ultracentrifugation. This was carried out as described in Chapter 2 using an *E. coli* MRE600 ribosome preparation as a control. Both *R. vannielii* 46S and 26S ribosomal subunits were found to be smaller than the respective *E. coli* 50S and 30S subunits.

Chapter 4

Results and Discussion

Chapter 4 Results and Discussion.

Examination and Cloning of Ribosomal DNA from *R. vannielii*.

4.0 Introduction.

The present day techniques of gene manipulation provide a powerful means by which to examine gene expression. Isolation of *R. vannielii* rDNA can facilitate sensitve means of detecting ribosomal transcripts and allow an examination of the role of regulatory DNA sequences. In addition, these studies provide an interesting insight into the physical arrangement of the rDNA of this organism.

4.1 Examination of the rDNA Gene Organization in *R. vannielii*.

4.1.1 Summary.

Both 16S and 23S rRNA coding sequences have been mapped on restriction digests of *R. vannielii* genomic DNA. The results indicated that *R. vannielii* has at least two rRNA operons (assuming the characteristic 5'.16S.23S.5S.3' format). However, the banding pattern obtained suggests that *R. vanneilii* may also have a number of additional ribosomal sequences.

4.1.2 Experimental Details.

R. vannielii genomic DNA was cut with restriction endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed at high stringency with either 16S, 23S or total rRNA. The resulting autoradiograph patterns are shown in figure 4.1 and summarized on table 4.1.

In assuming a conventional prokaryotic rRNA operon structure of 5.'16S.23S.5S.3', the two *Hind*III fragments of sizes 2.8 and 4.4 kb (shown to produce a strong signal with both 16S and 23S probes, table 4.1) would have been derived from the centre region of at least two different operons. The remaining *Hind*III fragments of sizes 4.7, 2.6 and 0.5 kb (shown to contain only 23S or 16S sequences) would have represented part of the operon only extending away from its core. It seems likely, therefore, that *R. vannielii* has at least two rRNA operons. Application of simular logic to the *Eco*RI rRNA fragments (table 4.1) suggested at least one rRNA operon is present.

However, a few bands were found to produce an uncharacteristically weak signal with both 16S and 23S probes. This observation posses interesting questions as to the organization/nature of the rDNA in *R. vannielii* and the possible existence of ribosomal pseudogenes cannot be overlooked. Further evidence for an atypical eubacterial rDNA structure in this organism is presented in section 4.2.

Figure 4.1.

Characterization of Ribosomal Sequences in Restriction Endonuculease Digests of *R. vannielii* Genomic DNA.

R. vannielii genomic DNA was restricted with the enzymes indicated, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with *R. vannielii* rRNA at 95% stringency as indicated.

Key to tracks:

A *R. vannielii* genomic DNA restricted with *Eco*RI

B *R. vannielii* genomic DNA restricted with *Hind*III

C Autoradiograph of track A probed with total rRNA

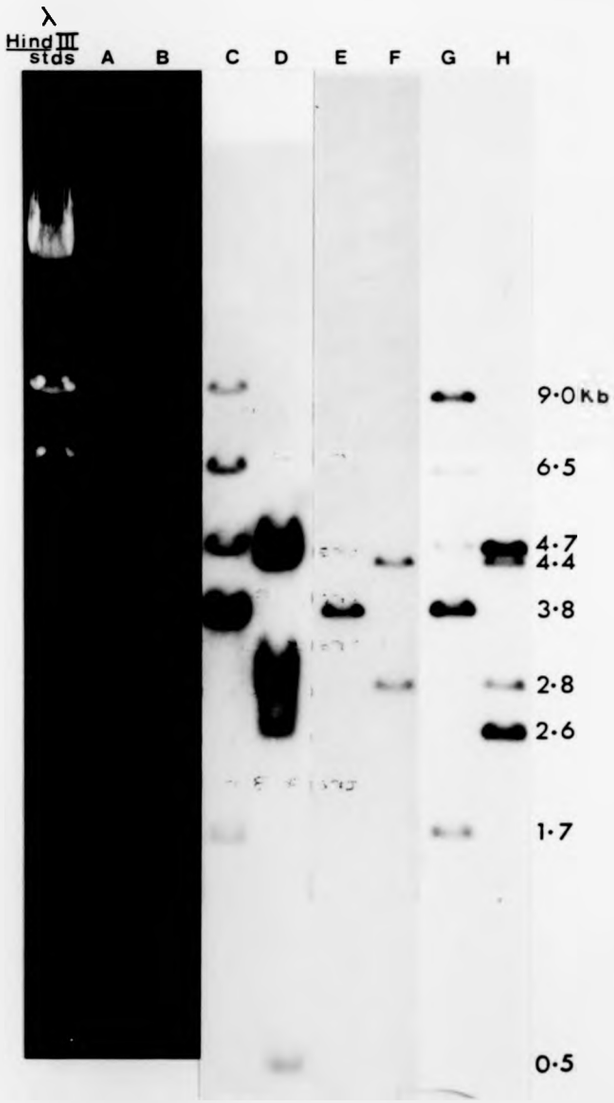
D Autoradiograph of track B probed with total rRNA

E Autoradiograph of track A probed with 23S rRNA

F Autoradiograph of track B probed with 23S rRNA

G Autoradiograph of track A probed with 16S rRNA

H Autoradiograph of track B probed with 16S rRNA



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Characterization of Ribosomal Sequences in Restriction Endonuculease Digests of *R. vannielii* Genomic DNA.

R. vannielii genomic DNA was restricted with the enzymes indicated, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with *R. vannielii* rRNA at 95% stringency as indicated.

Key to tracks:

A *R. vannielii* genomic DNA restricted with *EcoRI*

B *R. vannielii* genomic DNA restricted with *HindIII*

C Autoradiograph of track A probed with total rRNA

D Autoradiograph of track B probed with total rRNA

E Autoradiograph of track A probed with 23S rRNA

F Autoradiograph of track B probed with 23S rRNA

G Autoradiograph of track A probed with 16S rRNA

H Autoradiograph of track B probed with 16S rRNA

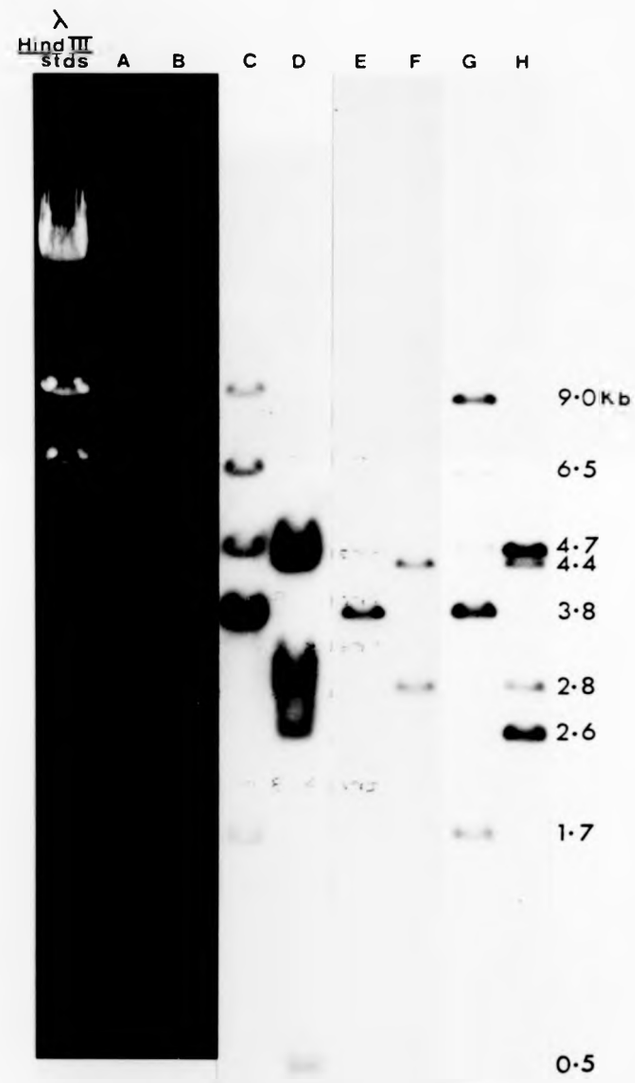


Table 4.1

Sizes of *R. vannielii* Genomic DNA Restriction Fragments That Show Homology to 23S and 16S rRNA.

This table summarizes the data given in figure 4.1.

Restriction Endonuclease	*Eco*R1		*Hind*III	
Homology with	16S	23S	16S	23S
Band sizes (kb)	9.0	-	4.7	-
	6.5	6.5	4.4	4.4
	4.7	4.7	2.8	2.8
	3.8	3.8	2.6	-
	1.7	-	-	0.5

4.2. Cloning and Characterization of rDNA Genes From *R. vannielii*.

4.2.1 Summary.

Two *R. vannielii* rDNA clones pC01 and pC02 have been isolated by rRNA/DNA homology from a selective gene library. pC01 and pC02 have *Eco*R1 inserts of 8.8 and 9.1 kbp respectively, these have been mapped and each shown to contain 16S rRNA coding sequences. The 16S rRNA coding regions show a good degree of homology with respect to restriction endonuclease recognition sites. The 16S rRNA coding sequences did not appear to be closely linked to any other rRNA genes, posing further interesting questions as to the organization of rDNA in *R. vannielii*.

4.2.2 Experimental Details.

A 9.0 kb *Eco*R1 fragment of *R. vannielii* genomic DNA, previously shown to contain only 16S rRNA coding sequences (see figure 4.1), was chosen for isolation. This fragment was selected to optimize the chances of isolating rRNA promoters as well as the 16S coding sequence. It was considered that a 9 kb fragment with only 16S sequences would contain at 8 kb of DNA to the 5' side of a conventional eubacterial rRNA operon and consequently contain the promoter region. The cloning strategy used is detailed in figure 4.2 and involved the construction of a selective gene bank in the *Eco*R1 site of pBR325, the resulting library being screened by hybridization.

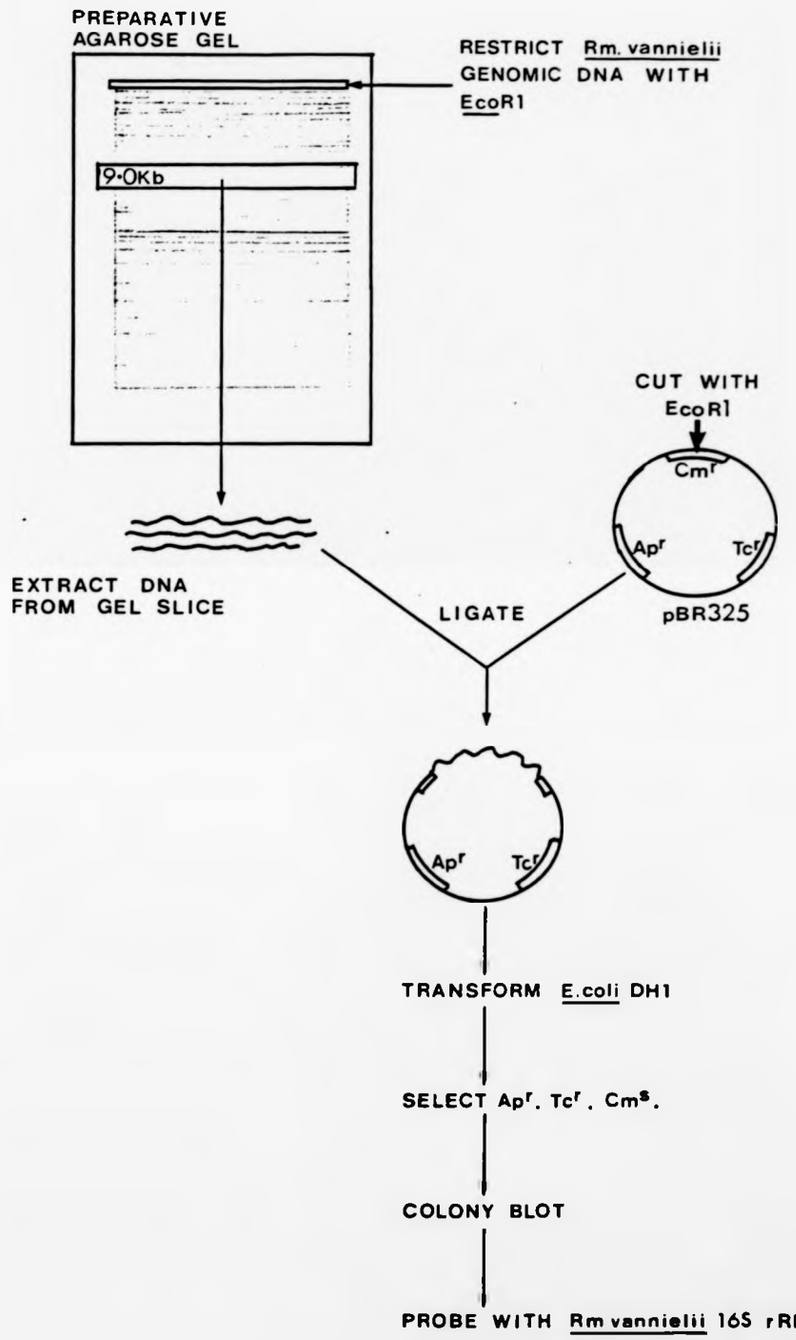
Due to the strong sequence homology that is found between prokaryotic rRNA sequences, measures were taken to optimize the autoradiograph signal of a positive clone over the background signal resulting from the seven *E. coli* DH1 rRNA operons. These involved the use of a high copy number vector whose copy number can be further increased by amplification with chloramphenicol. Screening of the library was carried out with a homologous *R. vannielii* 16S rRNA probe. The use of a homologous probe ensured maximum hybridization at high stringency and a purified 16S rRNA probe maximized specificity for a purely 16S rRNA target sequence. The resulting autoradiograph, figure 4.3., showes the two clones pCO1 and pCO2 clearly above the backgroud. Both pCO1 and pCO2 were later shown to contain rRNA sequences (figure 4.4).

In order to verify that both clones derived from *R. vannielii* genomic DNA and that no rearrangements had occurred during the cloning procedure, the following checks were made. *R. vannielii* DNA was restricted with both *Eco*R1 plus *Hind*III and *Eco*R1 plus *Pst*I, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with insert DNA from either pCO1 (figure 4.5) or pCO2 (figure 4.7). The resulting autoradiograph banding pattern was compared with that observed for the respective clones digested with the same pair of restriction endonucleases. All bands resulting from the cloned DNA's correlated, indicating that both clones had derived from *R. vannielii* DNA and had suffered no physical rearrangement.

Figure 4.2.

Cloning Scheme for *R. vannielii* rDNA.

The 9.0 Kb *Eco*R1 fragment from *R. vannielii* genomic DNA was chosen for isolation as shown.



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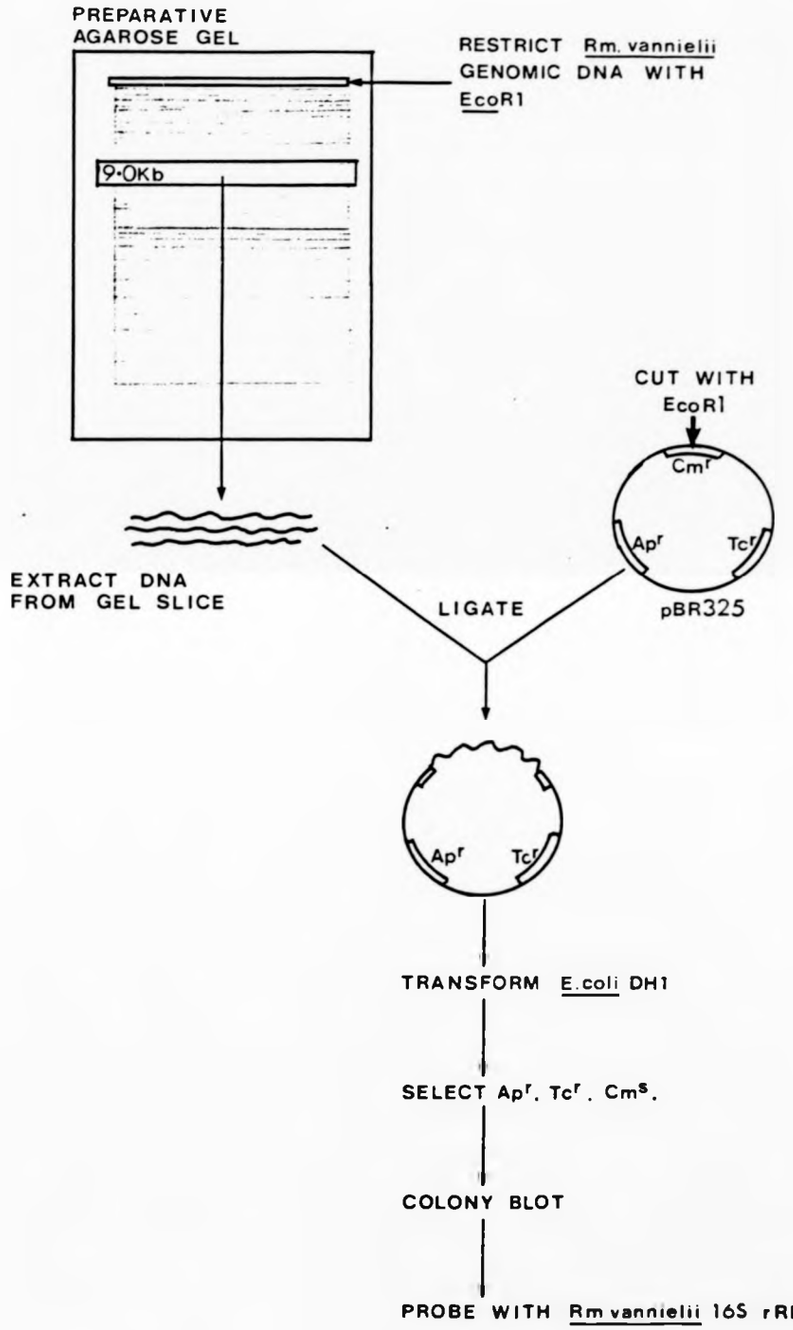
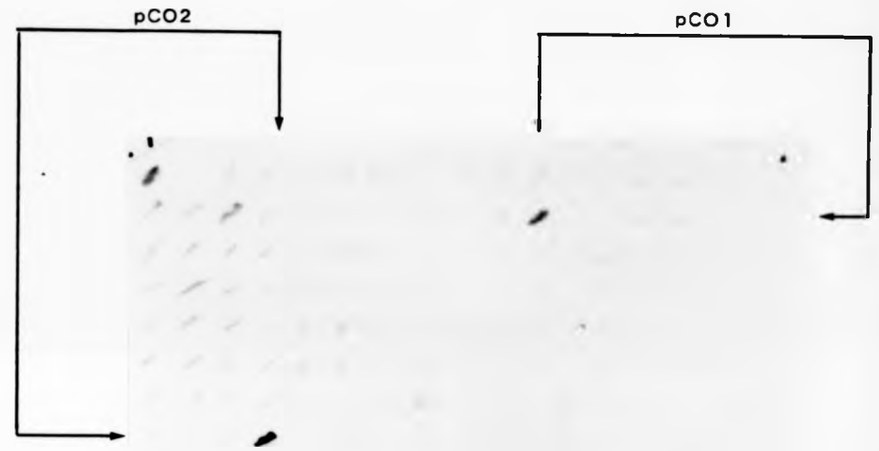


Figure 4.3.

Screening a Selective *R. vannielii* Gene Bank for 16S rRNA Sequences.

The cloning of *R. vannielii* rDNA was carried out as outlined in figure 4.2. The resulting colony blot was probed at 99 % stringency with end labelled *R. vannielii* 16S rRNA. The figure shows the two rRNA clones pCO1 and pCO2 on the resulting autoradiograph.



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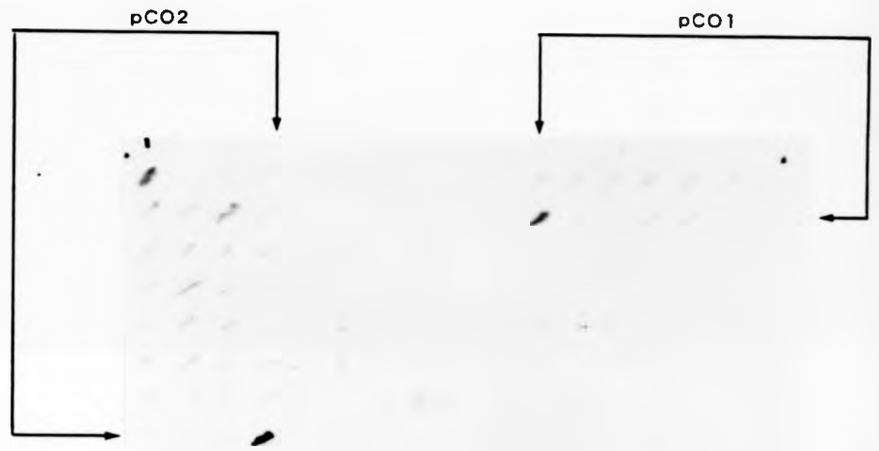


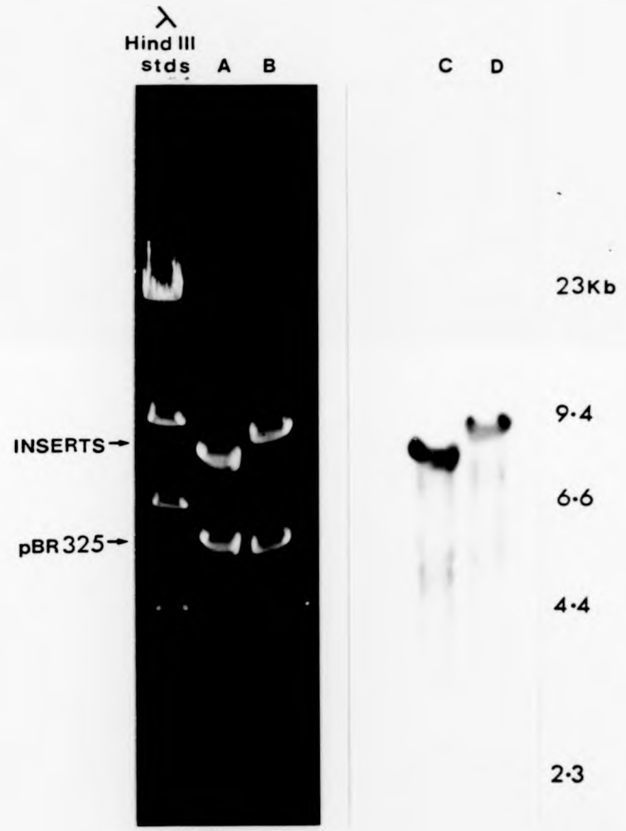
Figure 4.4.

Verification That pCO1 and pCO2 Have Inserts That Contain Ribosomal Sequences.

The clones pCO1 and pCO2 were isolated and restricted with *Eco*R1 to separate vectors from inserts and fractionated by agarose gel electrophoresis as shown. The resulting DNA bands were transfered by Southern blotting onto a nitrocellulose filter, probed with *R. vannielii* rRNA at 95 % stringency and subjected to autoradiography.

Key to tracks:

A	pCO1 restricted with *Eco*R1
B	pCO2 restricted with *Eco*R1
C	Autoradiograph of A probed with rRNA
D	Autoradiograph of B probed with rRNA



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Verification That pCO1 and pCO2 Have Inserts That Contain Ribosomal Sequences.

The clones pCO1 and pCO2 were isolated and restricted with *Eco*R1 to separate vectors from inserts and fractionated by agarose gel electrophoresis as shown. The resulting DNA bands were transfered by Southern blotting onto a nitrocellulose filter, probed with *R. vannielii* rRNA at 95 % stringency and subjected to autoradiography.

Key to tracks:

A	pCO1 restricted with *Eco*R1
B	pCO2 restricted with *Eco*R1
C	Autoradiograph of A probed with rRNA
D	Autoradiograph of B probed with rRNA

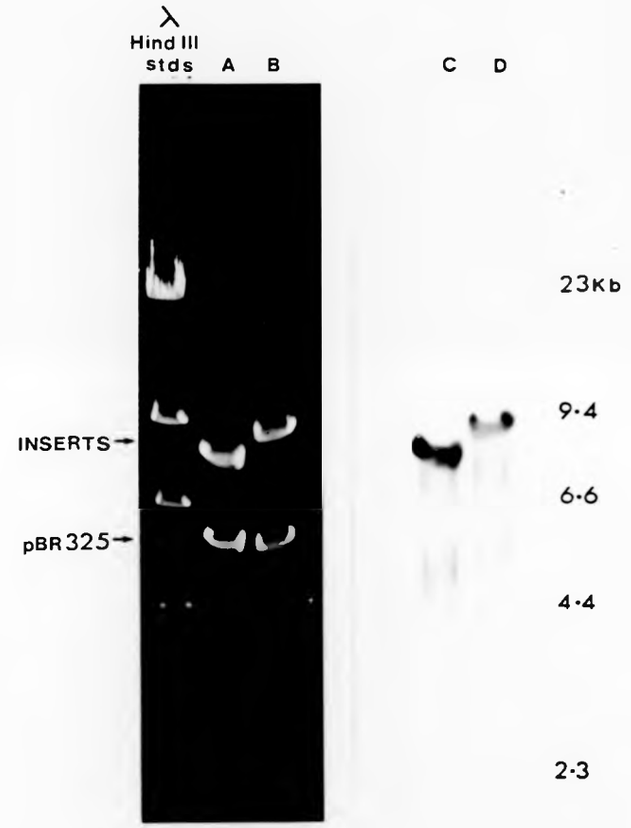


Figure 4.5.

Verification of the Origin and Continuity of the *R. vannielii* rDNA Clone pCO1.

R. vannielii genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter. This filter was then probed with a nick translated pCO1 insert at 95 % stringency.

Key:

A *R. vannielii* DNA restricted with *Eco*R1 & *Hind*III.
B Autoradiograph of A probed with the pCO1 insert.
C pCO1 restricted with *Eco*R1 & *Hind*III.

D *R. vannielii* DNA restricted with *Eco*R1 & *Pst*I.
E Autoradiograph of D probed with the pCO1 insert.
F pCO1 rstricted with *Eco*R1 and *Pst*I.

The banding pattern in tracks B and C and those in tracks E and F can be seen to correlate (with the omission of the vector bands).

(Note : please refer to figure 4.6 for an expansion of tracks B and E).

Figure 4.5.

Verification of the Origin and Continuity of the *R. vannielii* rDNA Clone pCO1.

R. vannielii genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis and transferred to a nitrocellulose filter. This filter was then probed with a nick translated pCO1 insert at 95 % stringency.

Key:

A *R. vannielii* DNA restricted with *Eco*R1 & *Hind*III.

B Autoradiograph of A probed with the pCO1 insert.

C pCO1 restricted with *Eco*R1 & *Hind*III.

D *R. vannielii* DNA restricted with *Eco*R1 & *Pst*I.

E Autoradiograph of D probed with the pCO1 insert.

F pCO1 rstricted with *Eco*R1 and *Pst*I.

The banding pattern in tracks B and C and those in tracks E and F can be seen to correlate (with the omission of the vector bands).

(Note : please refer to figure 4.6 for an expansion of tracks B and E).

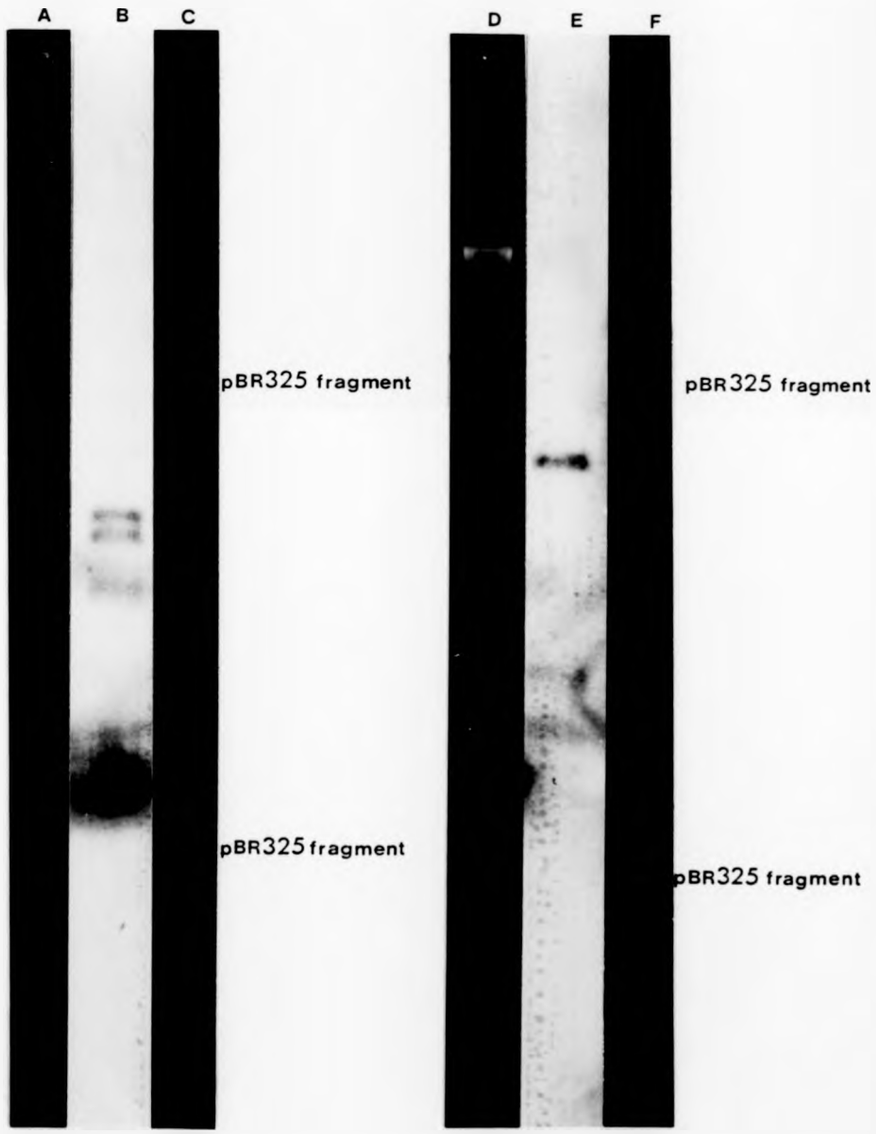


Figure 4.6.

Supplement to figure 4.5.

Figure 4.6.

Supplement to figure 4.5.

Figure 4.7.

Verification of the Origin and Continuity of the *R. vannielii* rDNA Clone pCO2.

R. vannielii genomic DNA was restricted as indicated, fractionated by agarose gel elctrophoresis and transferred to a nitrocellulose filter. This filter was then probed with a nick translated pCO2 insert at 95 % stringency.

Key:

A *R. vannielii* DNA restricted with *Eco*R1 & *Hind*III.
B Autoradiograph of A probed with the pCO2 insert.
C pCO2 restricted with *Eco*R1 & *Hind*III.

D *R. vannielii* DNA restricted with *Eco*R1 & *Pst*I.
E Autoradiograph of D probed with the pCO2 insert.
F pCO2 restricted with *Eco*R1 & *Pst*I.

The banding pattern in tracks B and C and those on tracks E and F can be seen to correlate (with the omission of the vector bands).

* This is not a valid band (compare with figure 4.9, track D) and is assumed to derive from non-specific restriction of pBR325.

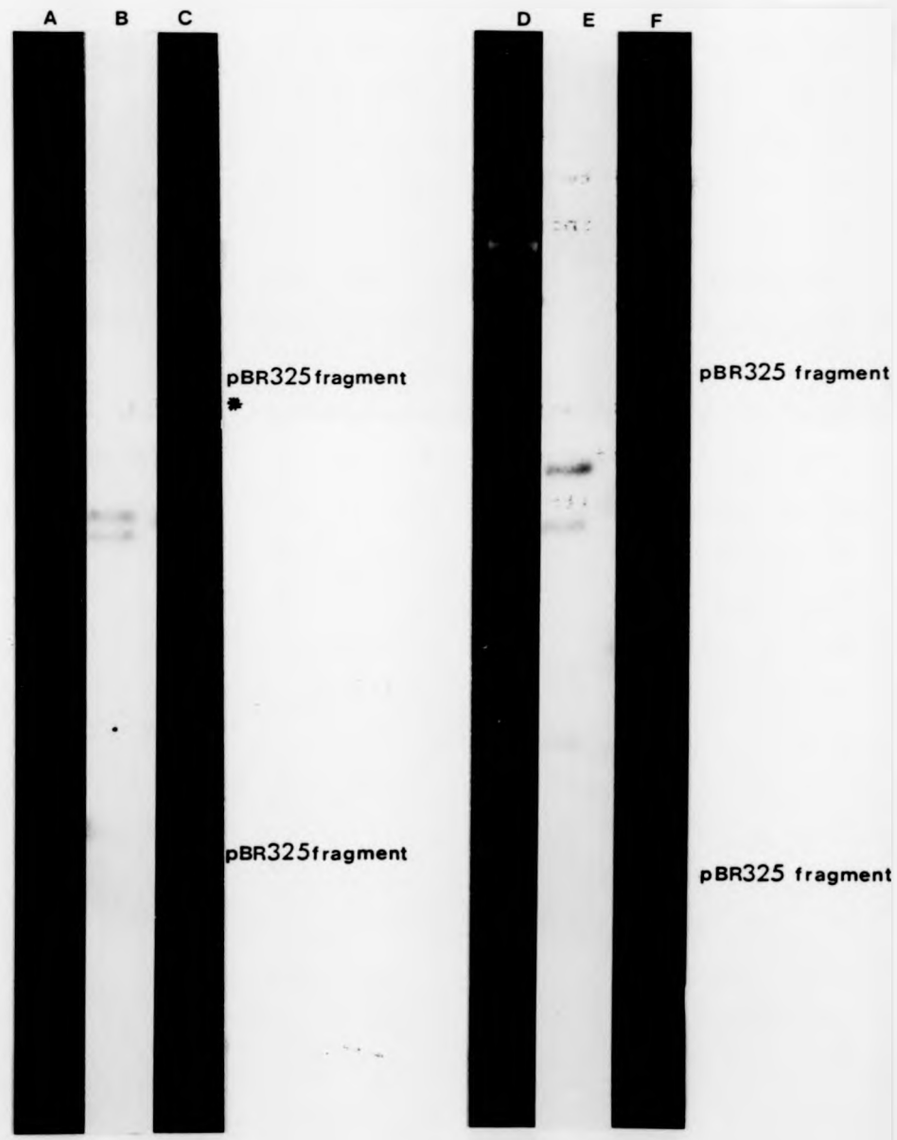


Figure 4.7.

Verification of the Origin and Continuity of the *R. vannielii* rDNA Clone pCO2.

R. vannielii genomic DNA was restricted as indicated, fractionated by agarose gel elctrophoresis and transferred to a nitrocellulose filter. This filter was then probed with a nick translated pCO2 insert at 95 % stringency.

Key:

A *R. vannielii* DNA restricted with *Eco*R1 & *Hind*III.

B Autoradiograph of A probed with the pCO2 insert.

C pCO2 restricted with *Eco*R1 & *Hind*III.

D *R. vannielii* DNA restricted with *Eco*R1 & *Pst*I.

E Autoradiograph of D probed with the pCO2 insert.

F pCO2 restricted with *Eco*R1 & *Pst*I.

The banding pattern in tracks B and C and those on tracks E and F can be seen to correlate (with the omission of the vector bands).

* This is not a valid band (compare with figure 4.9, track D) and is assumed to derive from non-specific restriction of pBR325.

pCO1 and pCO2 have been mapped with respect to several restriction endonucleases (figures 4.8, 4.9, 4.10 and summarized in figure 4.13). The position of the 16S coding sequence was determined by probing restriction digests of pCO1 and pCO2 with end labelled *R. vanielii* 16S rRNA. In both clones the 1.7 kb 16S rRNA coding sequence was found to span both a *SalI* and a *PstI* site and was positioned on this basis (figures 4.11 and 4.12).

The characterization of pCO1 and pCO2 is summarized in figure 4.13. pCO1 and pCO2 are 8.8 and 9.1 kb respectively and both contain 16S rRNA sequences. The two 16S coding sequences showed good homology in restriction site positioning (note: pCO2 was shown to have a *HindIII* site between *SalI* and *PstI* sites of the 16S rRNA coding sequence. This site is not shown as the insert was not fully mapped for this enzyme) with the exception of the *AvaI* site of pCO2. The closest linkage that the 16S rRNA genes of pCO1 and pCO2 can have to another rRNA gene is 2 kb. It may be that *R. vannielii* is unique among those eubacteria studied to date in the organisation of its ribosomal genes. All known eubacterial rDNA's are organized in closely linked operons with the characteristic gene sequence 5'.16S.23S.5S.3'. A variation has been shown for one archaebacterium, *Thermoplasma acidophilum* in which the single copies of each of the three rRNA genes are unlinked with at least 7.5 kbp separating the 16S and 23S genes (Tu & Zillig 1982). When shown, the distance between the eubacterial (and most archaebacterial) 16S and 23S rRNA genes

Figure 4.8.

Restriction Endonuclease Mapping of the *R. vannielii* rDNA Clone pCO1.

Key:

A	pBR322	restricted with *Hinf*I
B	λ DNA	restricted with *Hind*III
C	pCO1	restricted with *Eco*R1
D	pCO1	restricted with *Eco*R1 and *Hind*III
E	pCO1	restricted with *Hind*III
F	pCO1	restricted with *Eco*R1 and *Pst*I
G	pCO1	restricted with *Hind*III and *Pst*I
H	pCO1	restricted with *Pst*I
I	pCO1	restricted with *Eco*R1 and *Sal*I
J	pCO1	restricted with *Hind*III and *Sal*I
K	pCO1	restricted with *Pst*I and *Sal*I
L	pCO1	restricted with *Sal*I
M	as B	
N	as A	

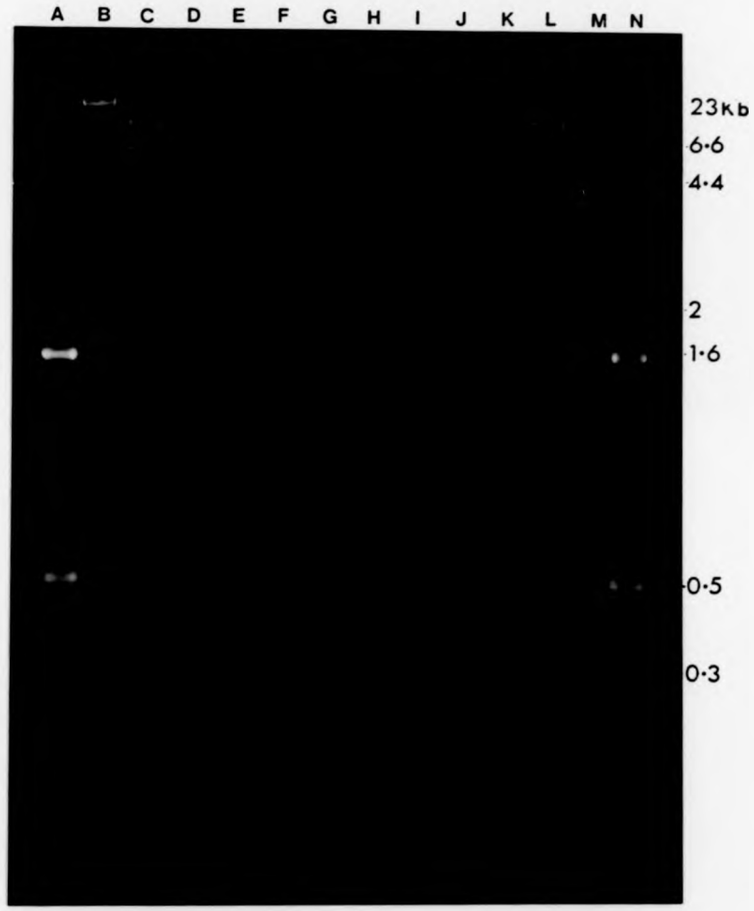


Figure 4.8.

Restriction Endonuclease Mapping of the *R. vannielii* rDNA Clone pCO1.

Key:

A	pBR322	restricted with *Hinf*I
B	λ DNA	restricted with *Hind*III
C	pCO1	restricted with *Eco*R1
D	pCO1	restricted with *Eco*R1 and *Hind*III
E	pCO1	restricted with *Hind*III
F	pCO1	restricted with *Eco*R1 and *Pst*I
G	pCO1	restricted with *Hind*III and *Pst*I
H	pCO1	restricted with *Pst*I
I	pCO1	restricted with *Eco*R1 and *Sal*I
J	pCO1	restricted with *Hind*III and *Sal*I
K	pCO1	restricted with *Pst*I and *Sal*I
L	pCO1	restricted with *Sal*I
M	as B	
N	as A	

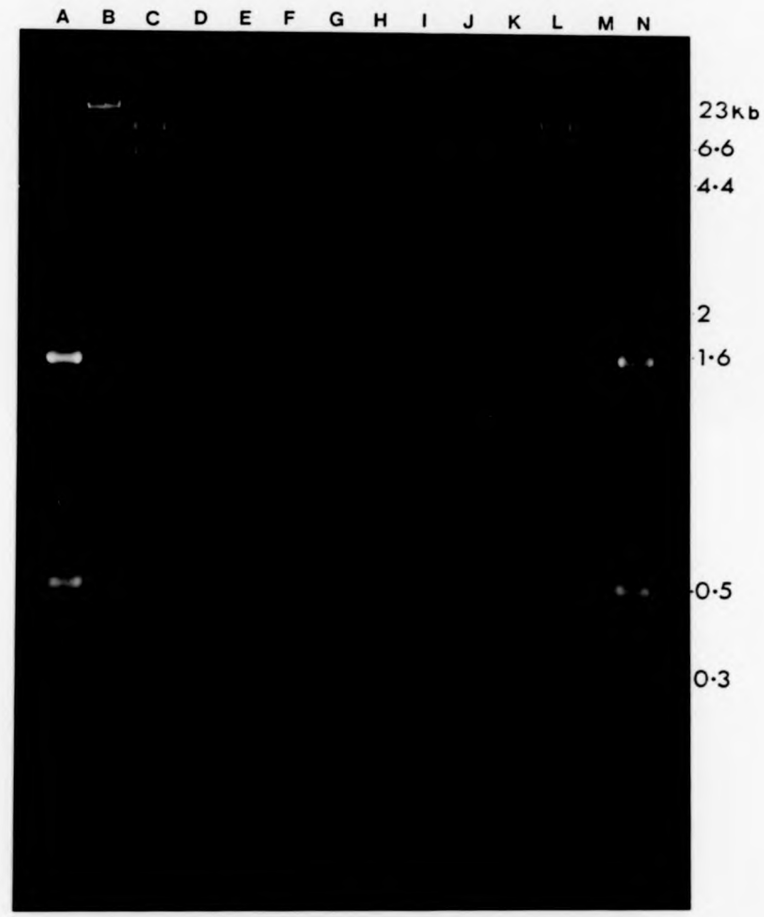


Figure 4.9.

Restriction Endonuclease Mapping of the *R. vannielii* rDNA Clone pCO2.

Key:

A	pBR322	restricted with *Hinf*I
B	λ DNA	restricted with *Hind*III
C	pCO2	restricted with *Eco*R1
D	pCO2	restricted with *Eco*R1 & *Hind*III
E	pCO2	restricted with *Hind*III
F	pCO2	restricted with *Eco*R1 & *Pst*I
G	pCO2	restricted with *Hind*III & *Pst*I
H	pCO2	restricted with *Pst*I
I	pCO2	restricted with *Eco*R1 & *Sal*I
J	pCO2	restricted with *Hind*III & *Sal*I
K	pCO2	restricted with *Pst*I & *Sal*I
L	pCO2	restricted with *Sal*I
M	as B	
N	as A	

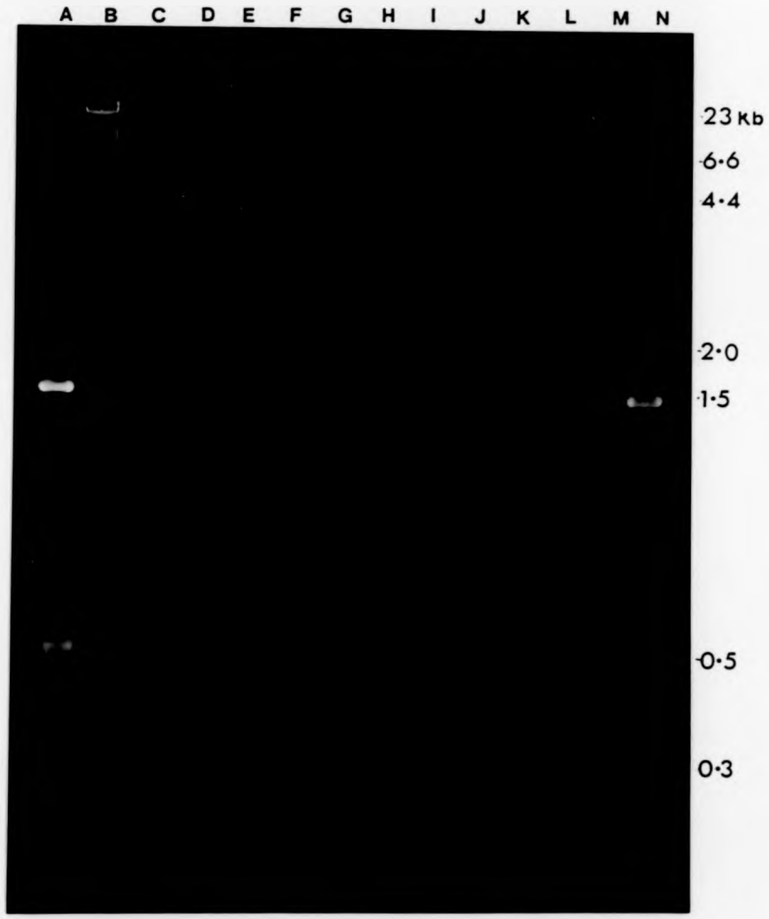


Figure 4.9.

Restriction Endonuclease Mapping of the *R. vannielii* rDNA Clone pCO2.

Key:

A	pBR322 restricted with *Hinf*I
B	λ DNA restricted with *Hind*III
C	pCO2 restricted with *Eco*R1
D	pCO2 restricted with *Eco*R1 & *Hind*III
E	pCO2 restricted with *Hind*III
F	pCO2 restricted with *Eco*R1 & *Pst*I
G	pCO2 restricted with *Hind*III & *Pst*I
H	pCO2 restricted with *Pst*I
I	pCO2 restricted with *Eco*R1 & *Sal*I
J	pCO2 restricted with *Hind*III & *Sal*I
K	pCO2 restricted with *Pst*I & *Sal*I
L	pCO2 restricted with *Sal*I
M	as B
N	as A

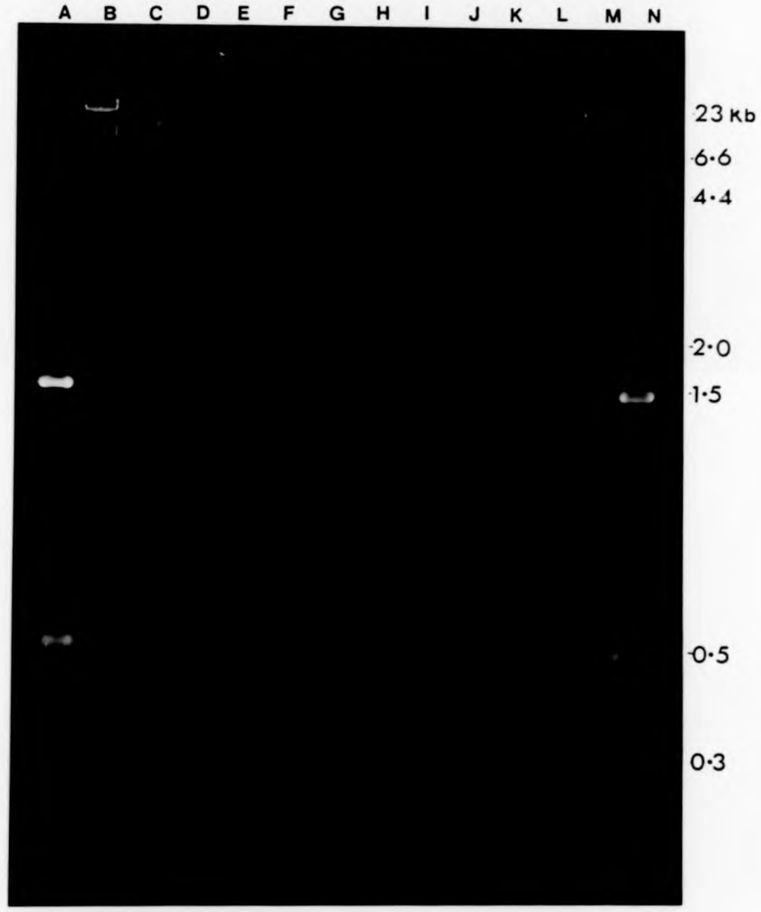


Figure 4.10.

Restriction Endonuclease Mapping of the *R. vannielii* rDNA Clones pCO1 and pCO2.

Key:

A	pCO1	restricted with *Ava*I
B	pCO1	restricted with *Ava*I and *Eco*R1
C	pCO1	restricted with *Ava*I and *Hind*III
D	pCO1	restricted with *Ava*I and *Pst*I
E	pCO1	restricted with *Ava*I and *Sal*I
F	λ	restricted with *Hind*III
G	pBR322	restricted with *Hinf*I
H	pCO2	restricted with *Ava*I
I	pCO2	restricted with *Ava*I and *Eco*R1
J	pCO2	restricted with *Ava*I and *Hind*III
K	pCO2	restricted with *Ava*I and *Pst*I
L	pCO2	restricted with *Ava*I and *Sal*I

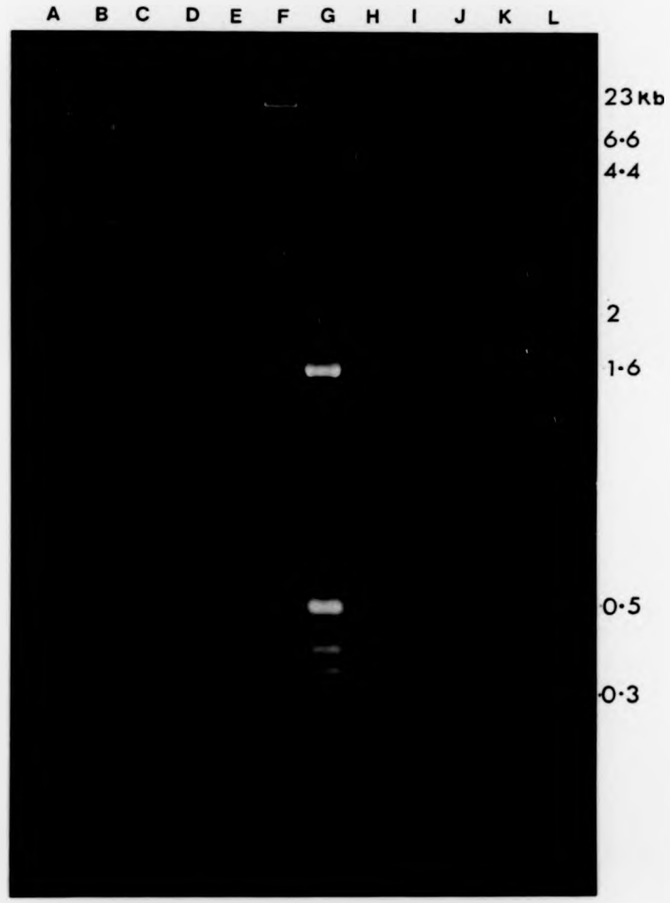


Figure 4.10.

Restriction Endonuclease Mapping of the *R. vannielii* rDNA Clones pCO1 and pCO2.

Key:

A	pCO1	restricted with *Ava*I
B	pCO1	restricted with *Ava*I and *Eco*R1
C	pCO1	restricted with *Ava*I and *Hind*III
D	pCO1	restricted with *Ava*I and *Pst*I
E	pCO1	restricted with *Ava*I and *Sal*I
F	λ	restricted with *Hind*III
G	pBR322	restricted with *Hinf*I
H	pCO2	restricted with *Ava*I
I	pCO2	restricted with *Ava*I and *Eco*R1
J	pCO2	restricted with *Ava*I and *Hind*III
K	pCO2	restricted with *Ava*I and *Pst*I
L	pCO2	restricted with *Ava*I and *Sal*I

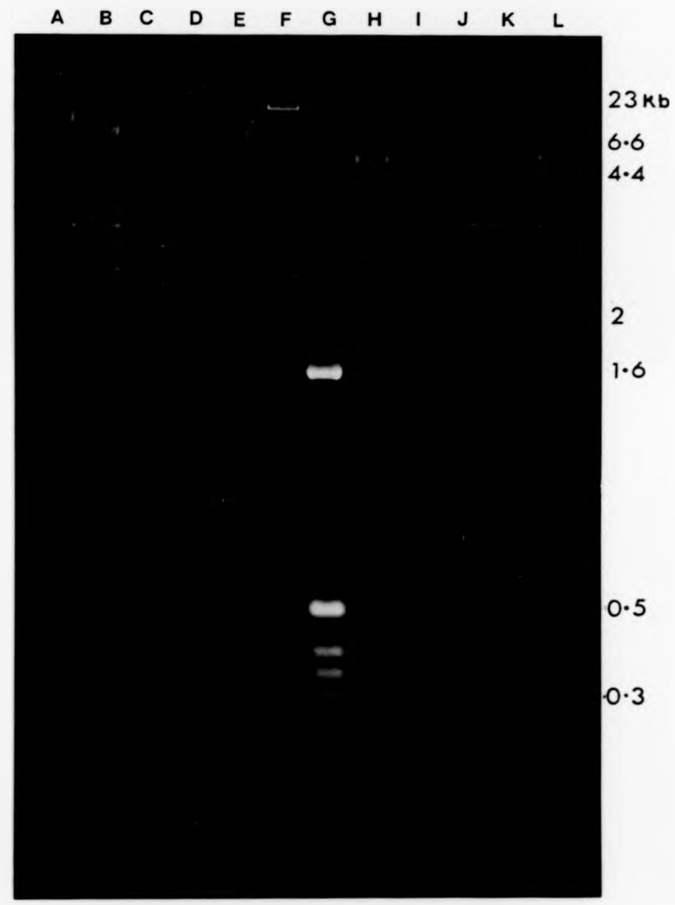


Figure 4.11.

Positioning the 16S rRNA Coding Sequence on the *R. vannielii* rDNA Clone pCO1.

Figure 4.11(a).

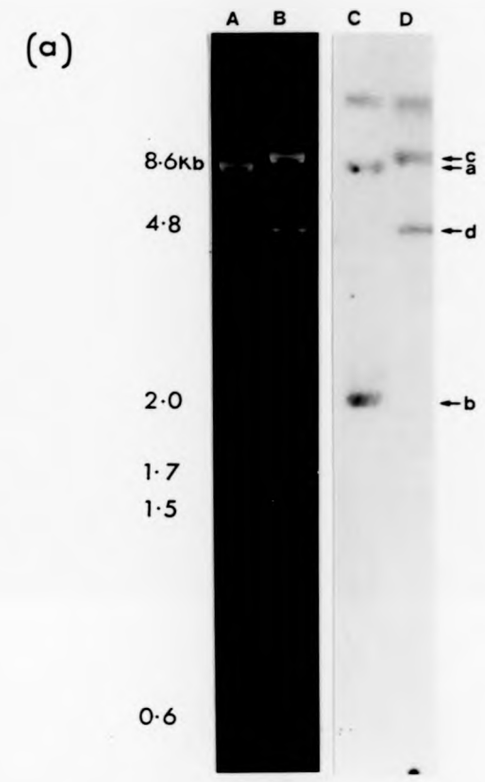
pCO1 was restricted with the enzymes indicated, fractionated by agarose gel electrophoresis and transferred by Southern blotting to a nitrocellulose filter. The resulting filter was probed with *R. vannielii* 16S rRNA at 95 % stringency and subjected to autoradiography.

Key to tracks:

A	pCO1 restricted with *PstI*
B	pCO1 restricted with *SalI*
C	Autoradiograph of A probed with 16S rRNA
D	Autoradiograph of B probed with 16S rRNA

Figure 4.11(b).

This diagram summarizes the data from figure 4.11 (a) and positions those bands shown to contain 16S rDNA sequences (designated a, b, c, and d) on a linearized map of pCO1 (refer to figure 4.13 for a detailed map).



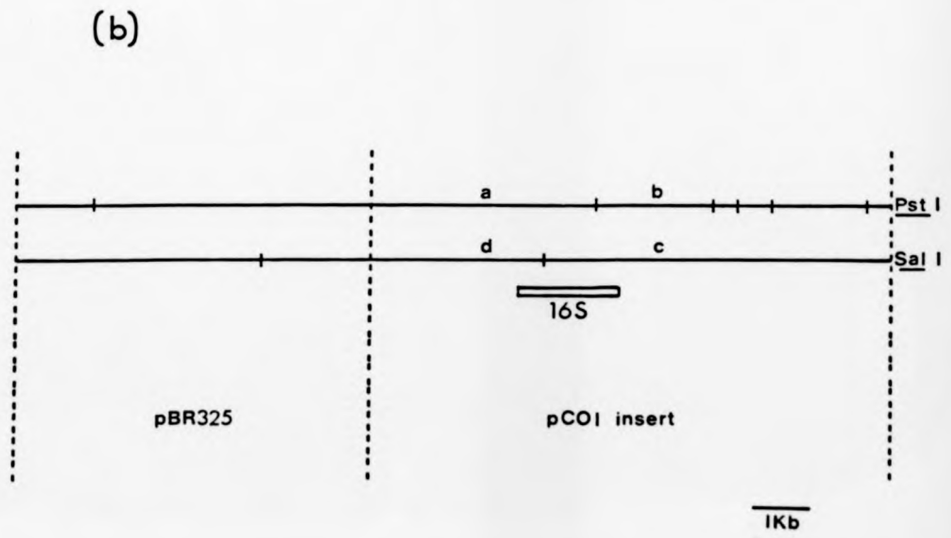


Figure 4.11.

Positioning the 16S rRNA Coding Sequence on the *R. vannielii* rDNA Clone pCO1.

Figure 4.11(a).

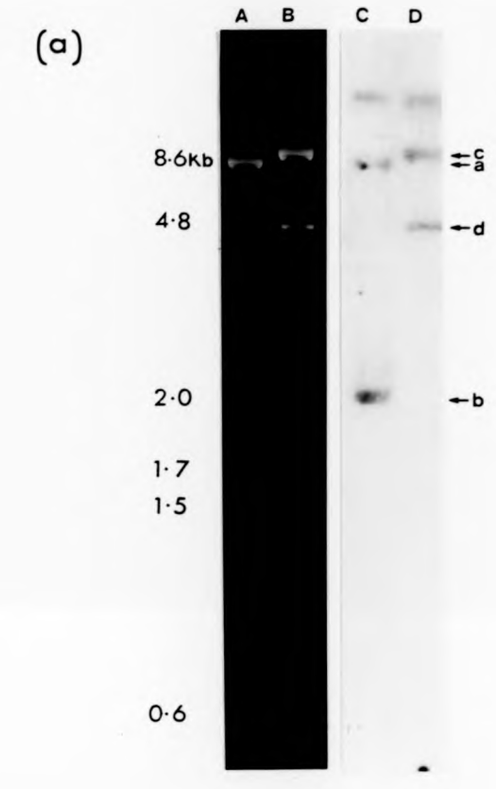
pCO1 was restricted with the enzymes indicated, fractionated by agarose gel electrophoresis and transferred by Southern blotting to a nitrocellulose filter. The resulting filter was probed with *R. vannielii* 16S rRNA at 95 % stringency and subjected to autoradiography.

Key to tracks:

A	pCO1 restricted with *Pst*I
B	pCO1 restricted with *Sal*I
C	Autoradiograph of A probed with 16S rRNA
D	Autoradiograph of B probed with 16S rRNA

Figure 4.11(b).

This diagram summarizes the data from figure 4.11 (a) and positions those bands shown to contain 16S rDNA sequences (designated a, b, c, and d) on a linearized map of pCO1 (refer to figure 4.13 for a detailed map).



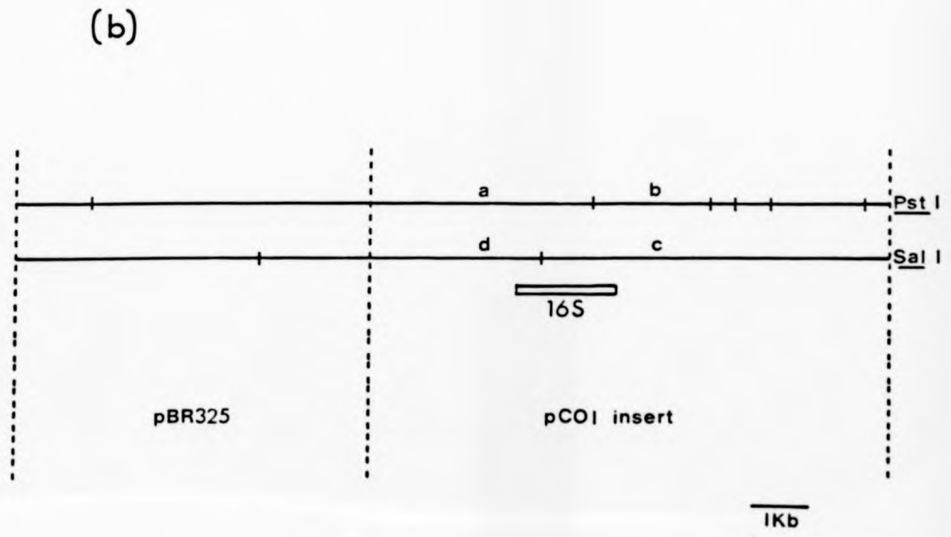


Figure 4.12.

Positioning of the 16S rRNA Coding Sequences on the *R. vannielii* rDNA clone pCO2.

Figure 4.12(a).

pCO2 was restricted with the enzymes indicated, fractionated by agarose gel electrophoresis and transferred by Southern blotting to a nitrocellulose filter. The resulting filter was probed with *R. vannielii* 16S rRNA at 95 % stringency and subjected to autoradiography.

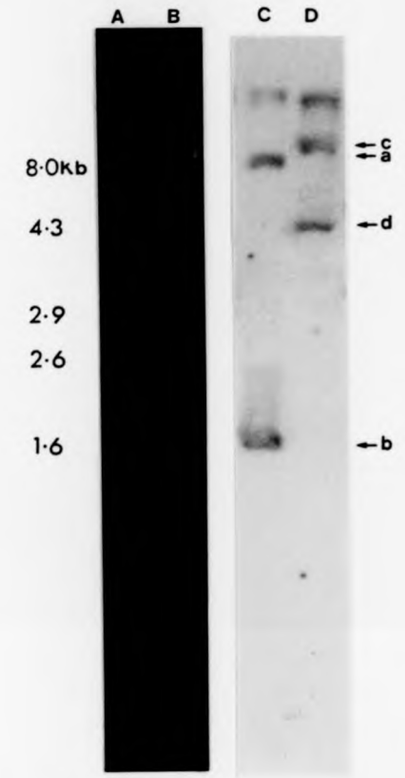
Key to tracks:

A	pCO2 restricted with *Pst*I
B	pCO2 restricted with *Sal*I
C	Autoradiograph of A probed with 16S rRNA
D	Autoradiograph of B probed with 16S rRNA

Figure 4.12(b).

This diagram summarizes the data from figure 4.12 (a) and positions those bands shown to contain rDNA sequences (designated a, b, c, and d) on a linearized map of pCO2 (refer to figure 4.13 for a detailed map).

(a)



(b)

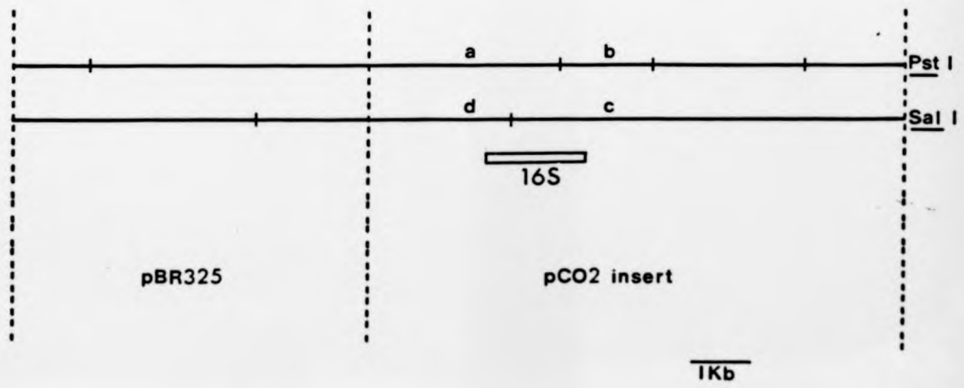


Figure 4.12.

Positioning of the 16S rRNA Coding Sequences on the *R. vannielii* rDNA clone pCO2.

Figure 4.12(a).

pCO2 was restricted with the enzymes indicated, fractionated by agarose gel electrophoresis and transferred by Southern blotting to a nitrocellulose filter. The resulting filter was probed with *R. vannielii* 16S rRNA at 95 % stringency and subjected to autoradiography.

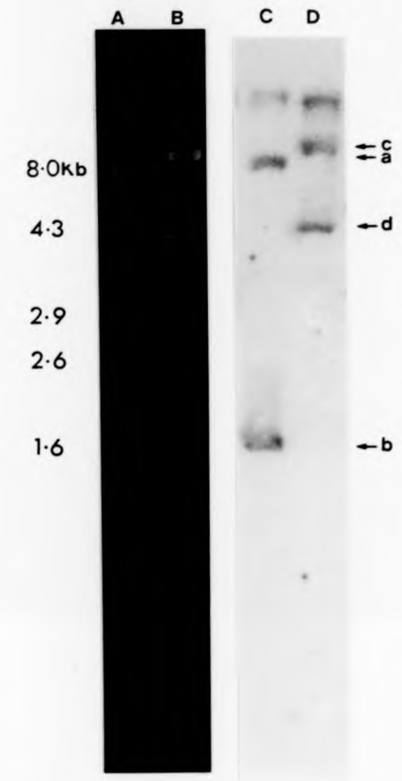
Key to tracks:

A	pCO2 restricted with *Pst*I
B	pCO2 restricted with *Sal*I
C	Autoradiograph of A probed with 16S rRNA
D	Autoradiograph of B probed with 16S rRNA

Figure 4.12(b).

This diagram summarizes the data from figure 4.12 (a) and positions those bands shown to contain rDNA sequences (designated a, b, c, and d) on a linearized map of pCO2 (refer to figure 4.13 for a detailed map).

(a)



(b)

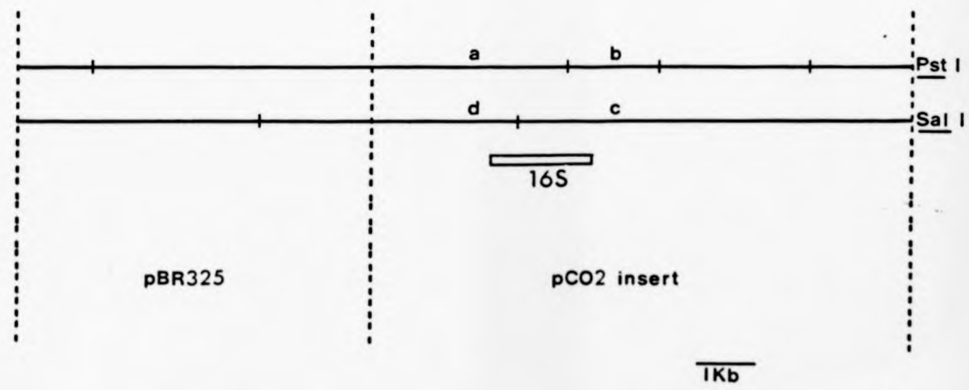
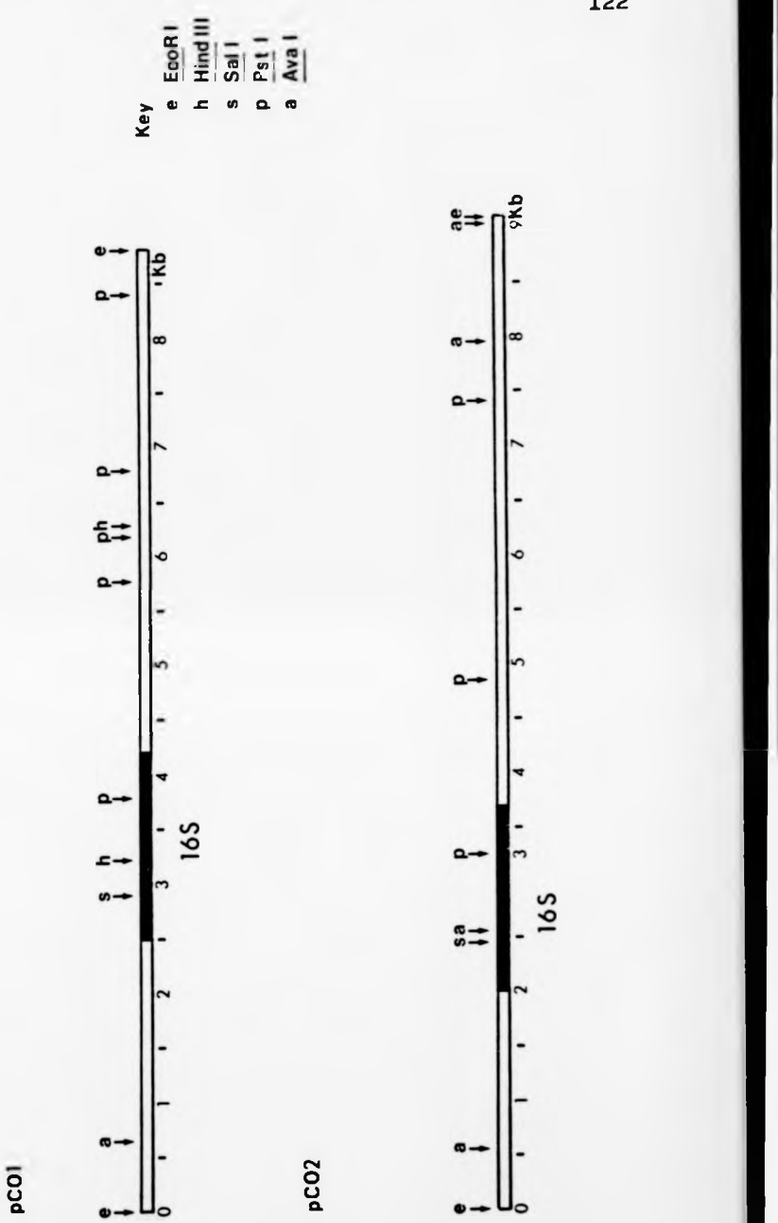


Figure 4.13.

Maps of the Inserts of the *R. vannielii* rDNA Clones pCO1 and pCO2.

The maps are drawn in a compatible orientation to that conventionally used for the host vector pBR325.



is in the order of hundreds of base pairs, often encoding a tRNA.

Unfortunately it is not known if the 16S genes of pCD1 and pCD2 are expressed or form a functional component of the rDNA of *R. vannielii*. However, these observations further add to the idea that the rDNA organization of this organism differs from previously studied eubacteria. It would be of great interest to examine the organization of *R. vannielii* ribosomal genes in more detail.

4.3 Search for Homology Between r-Proteins Genes from *R. vannielii* and *E. coli*.

4.3.1 Summary.

R. vannielii genomic DNA was probed with the genes for L7/12, L10, L1 and L11 ribosomal proteins from *E. coli*. No detectable homology was found.

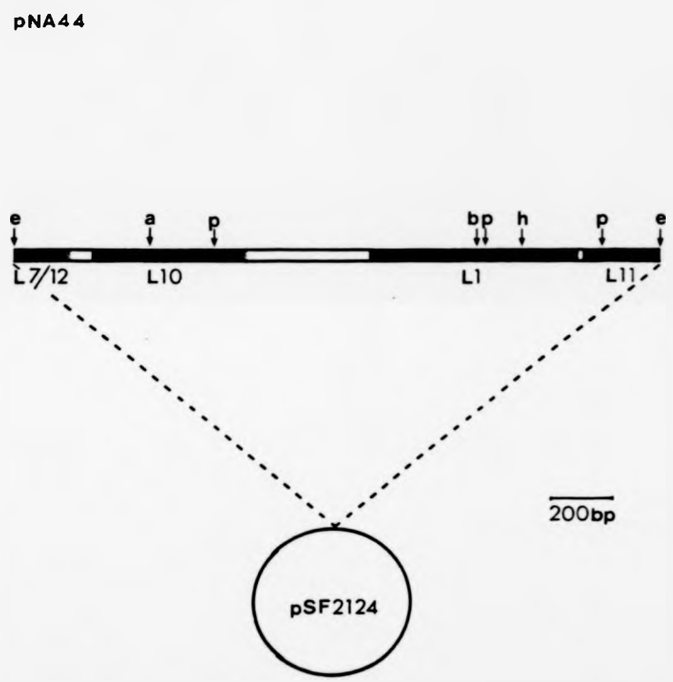
4.3.2 Experimental Details.

R. vannielii and *E. coli* HB101 genomic DNA was restricted, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose. The resulting filters were probed at 50 % stringency with the pNA44 (figure 4.14) insert containing *E. coli* L7/12, L10, L1 and L11 ribosomal protein genes. No detectable homology was found (figure 4.15)

Figure 4.14.

Map of the Plasmid pNA44 Which Carries the *E. coli* r-Protein Genes L7/12, L10, L1 and L11.

The insert of this plasmid was used to examine *R. vannielii* genomic DNA for inter-species r-protein gene homology (see figure 4.15). pSF2124 was constructed by Nicolaidis and Hayward (unpublished data) and the subcloning of the pNA44 region from $rif^{d}18$ was also carried out in the same laboratory (pNA44 was a gift from Dr. M. Ryan).



Key
- a Ava I
- b Bgl II
- e EcoRI
- h Hind III
- p Pst I

Figure 4.15.

Examination of *R. vannielii* Genomic DNA for Homology with *E. coli* r-Protein Gene Sequences.

R. vannielii and *E. coli* HB101 genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose filters. These filters were then probed with the pNA44 insert (see figure 4.14) and subjected to autoradiography.

Key:

(a) and (c) show ethidium bromide stained agarose gels and (b) and (d) show their respective autoradiographs.

A	*R. vannielii* DNA restricted with *Eco*R1
B	*R. vannielii* DNA restricted with *Hind*III
C	*R. vannielii* DNA restricted with *Sal*I
D	λ DNA restricted with *Hind*III
E	*R. vannielii* DNA restricted with *Eco*R1
F	*R. vannielii* DNA restricted with *Hind*III
G	*R. vannielii* DNA restricted with *Sal*I
H	λ DNA restricted with *Hind*III
I	*E.coli* HB101 DNA restricted with *Eco*R1

No interspecies homology was observed between the *E. coli* r-protein sequences and *R. vannielii* genomic DNA as shown in autoradiograph (b) 50 % stringency. Autoradiograph (c) results from 99 % stringency conditions to clarify the pNA44/*E. coli* control.

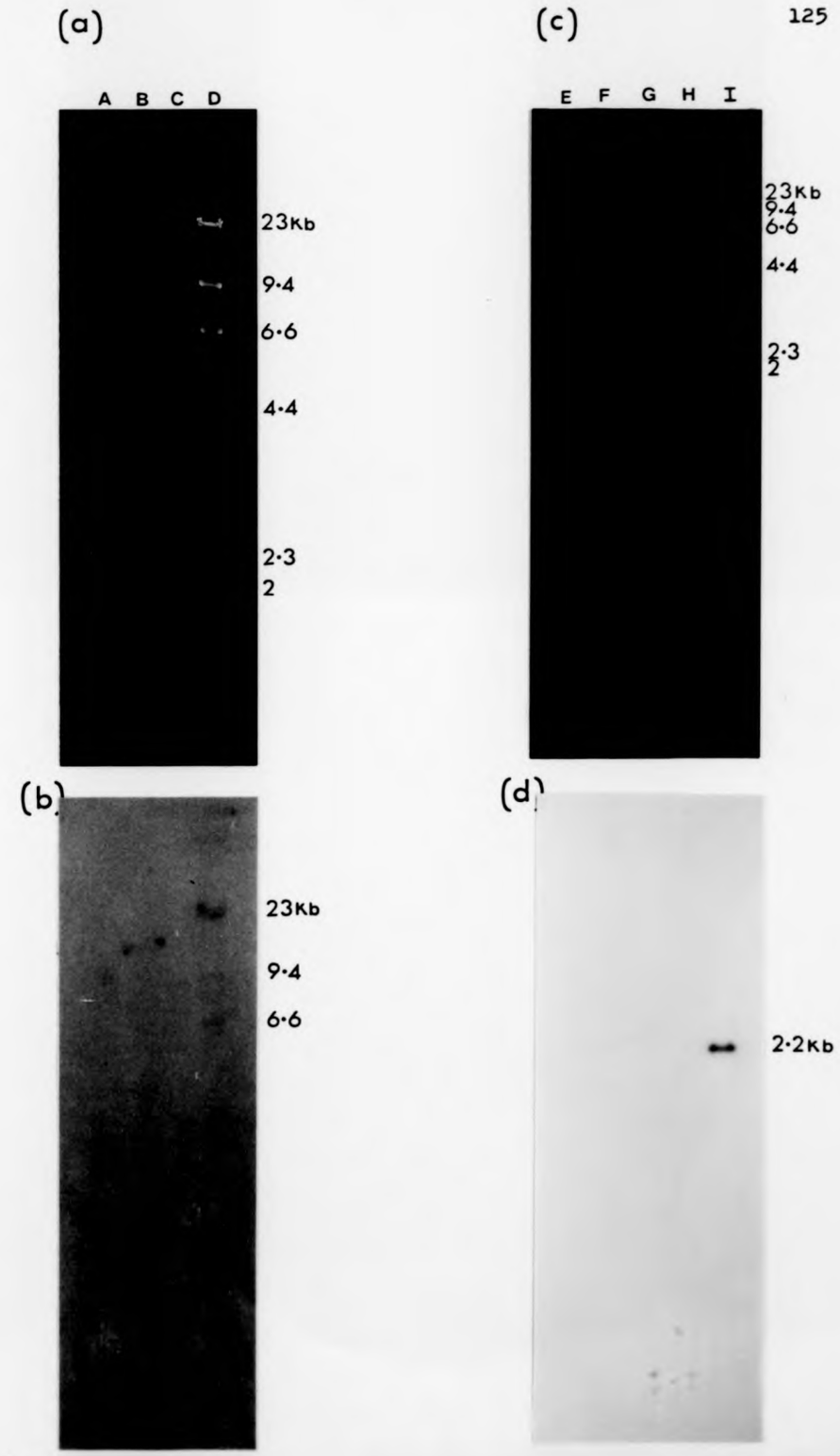


Figure 4.15.

Examination of *R. vannielii* Genomic DNA for Homology with *E. coli* r-Protein Gene Sequences.

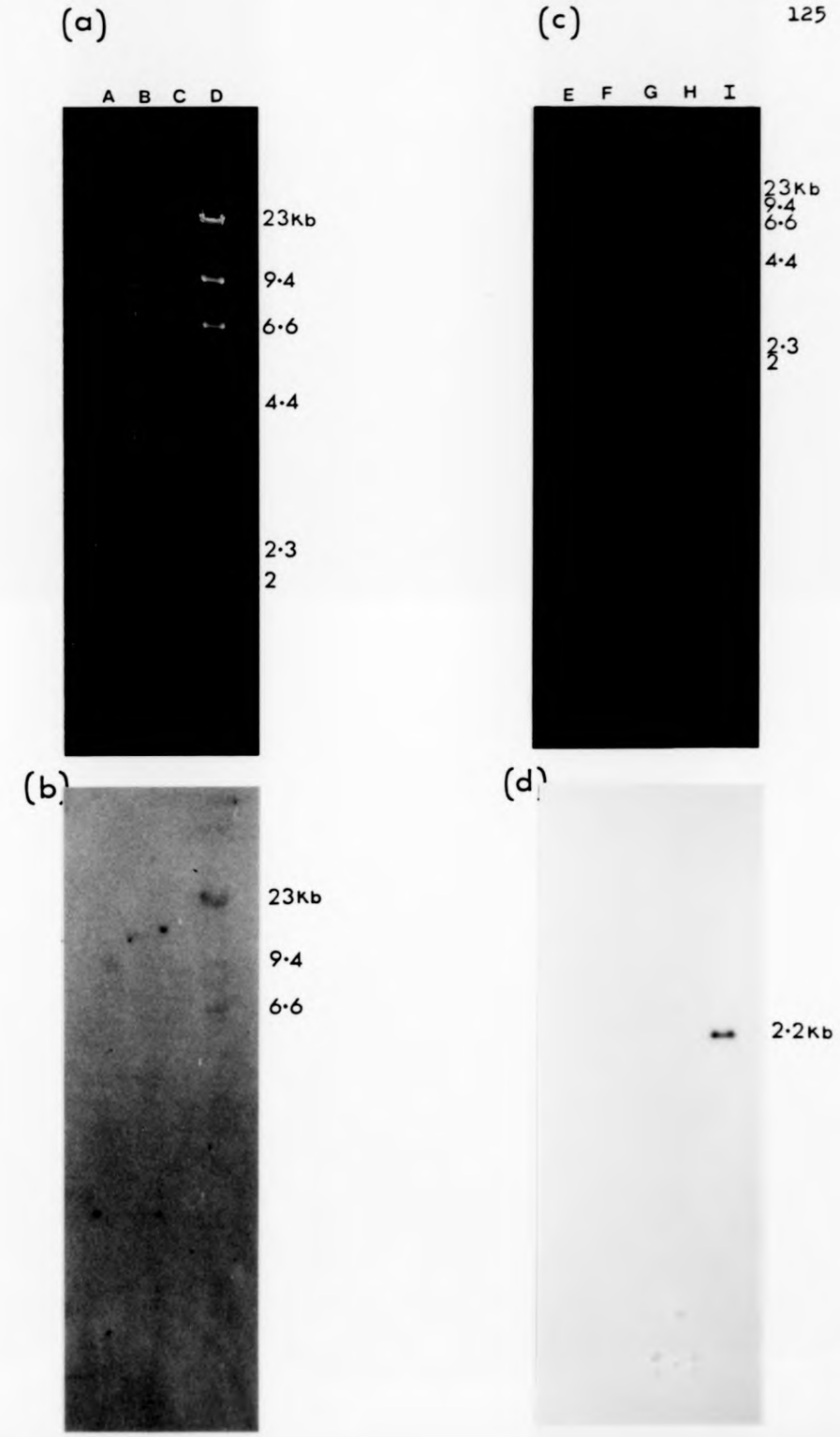
R. vannielii and *E. coli* HB101 genomic DNA was restricted as indicated, fractionated by agarose gel electrophoresis and Southern blotted onto nitrocellulose filters. These filters were then probed with the pNA44 insert (see figure 4.14) and subjected to autoradiography.

Key:

(a) and (c) show ethidium bromide stained agarose gels and (b) and (d) show their respective autoradiographs.

A	*R. vannielii* DNA restricted with *Eco*R1
B	*R. vannielii* DNA restricted with *Hind*III
C	*R. vannielii* DNA restricted with *Sal*I
D	λ DNA restricted with *Hind*III
E	*R. vannielii* DNA restricted with *Eco*R1
F	*R. vannielii* DNA restricted with *Hind*III
G	*R. vannielii* DNA restricted with *Sal*I
H	λ DNA restricted with *Hind*III
I	*E.coli* HB101 DNA restricted with *Eco*R1

No interspecies homology was observed between the *E. coli* r-protein sequences and *R. vannielii* genomic DNA as shown in autoradiograph (b) 50 % stringency. Autoradiograph (c) results from 99 % stringency conditions to clarify the pNA44/*E. coli* control.



Chapter 5

Results and Discussion

Chapter 5 Results and discussion.

Examination of Regulatory Mechanisms That May Function in Modulating Ribosome Synthesis in *R. vannielii*.

5.0 Introduction.

The results in section 3.3.4 are indicative of a nearly ten-fold reduction in the rate of incorporation of ^{35}S-methionine into the ribosomes of the dark-inhibited *R. vannielii* swarmer cell in comparison with its maturing counterpart. The fact that synthesis of r-protein occurs at all in this non-growing cell points to a turnover of ribosomes, though the rate of r-protein synthesis itself must be reduced in order to account for the much reduced level of ^{35}S-methionine incorporation. By analogy with the control of ribosome synthesis in *E. coli* (Nomura 1986), rRNA synthesis was also expected to be either much reduced or absent. In this chapter, possible regulatory mechanisms of ribosome synthesis are examined. The stringent response is a well studied modulator of ribosome synthesis in the prokaryotes and is characterized by an increase in the levels of highly phosphorylated nucleotides (HPN's). This response is known to be triggered by amino acid deprivation but can also be stimulated in photosynthetic organisms by light deprivation (Mann *et al* 1975, Campbell and Lueking 1983). For this reason it is important to examine *R. vannielii* for HPN's with respect to both cell cycle and growth conditions.

5.1 Examination of the Levels of Highly Phosphorylated Nucleotides During the Cell Cycle of *R. vannielii*.

5.1.1 Summary.

The levels of phosphorylated nucleotides were examined through the first four hours of swarmer cell maturation and reproduction. High levels of ATP were maintained throughout and appeared invarient. GTP levels were found at a lower level but also apeared invariant. The HPN's ppGpp and pppGpp were both present in *R. vanneilii* at very low levels which remained constant, both in the transiently inhibited swarmer cell and throughout its stages of maturation and reproduction During prolonged inhibition in the dark, a reduction in the levels of all the phosphorylated nucleotides was observed. A small increase in the level of pppGpp was observed when stalked cells were transferred to incubation in the dark. The results indicate that a stringent response is not a significant factor in the inhibition of swarmer cell maturation.

5.1.2 Experimental Details

A 100 ml batch culture of *R. vannielii* was labelled throughout growth with 50 μCi ml^{-1} ^{32}P-orthophosphate and the swarmer cells selected during the late exponential phase of growth. The resulting cells were either allowed to differentiate (figure 5.1 and 5.3) or were inhibited from initiating maturation by incubation in the dark (figure 5.2). Throughout the experiment samples were taken and prepared for

thin layer chromatography. The resulting chromatographs were subjected to autoradiography.

The phosphorylated nucleotides shown in figures 5.1, 5.2 and 5.3 were identified by comparison with the *E. coli* standards and published RF values.

Figure 5.1 shows labelled phosphorylated nucleotides in a differentiating swarmer cell population. The 0 hour sample represented an inhibited swarmer cell population in the dark just prior to initiation of maturation by incubation in the light. Very low levels of both ppGpp and pppGpp were apparent and showed no variation throughout differentiation. In addition no variation in GTP levels was evident. For this reason it was considered that the stringent response has no direct relationship with the physiological state of the inhibited swarmer cell.

Figure 5.2 shows the situation for inhibited swarmer cells during prolonged incubation in the dark. It can be seen that the levels of all phosphorylated nucleotides gradually decrease over a four hour period as this cell has means to replenish these energy pools.

In summary, it is tempting to speculate that the inhibited swarmer cell is inactivated in terms of growth related processes by an as yet undefined mechnism that supercedes the requirement for a stringent response.

It has been shown, however, that in the photosynthetic

Figure 5.1.

Examination of *R. vannielii* Swarmer Cells for the Presence of Highly Phosphorylated Nucleotides During Differentiation.

Batch cultures of *R. vannielii* were labelled throughout growth with 50 μCi ml^{-1} ^{32}P-orthophosphate and swarmer cells were selected during the late exponential phase of growth. The resulting cells were allowed to develop and were sampled at the times indicated. Samples were prepared for thin layer chromatography and autoradiography as described in Chapter 2. This figure shows the resulting autoradiograph.

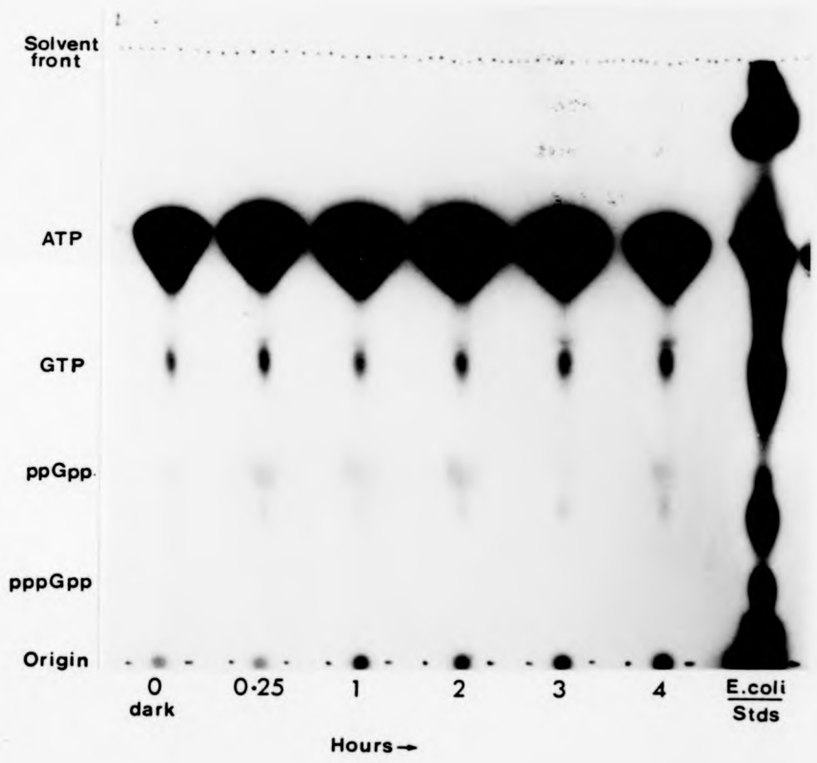


Figure 5.1.

Examination of *R. vannielii* Swarmer Cells for the Presence of Highly Phosphorylated Nucleotides During Differentiation.

Batch cultures of *R. vannielii* were labelled throughout growth with 50 μCi ml^{-1} ^{32}P-orthophosphate and swarmer cells were selected during the late exponential phase of growth. The resulting cells were allowed to develop and were sampled at the times indicated. Samples were prepared for thin layer chromatography and autoradiography as described in Chapter 2. This figure shows the resulting autoradiograph.

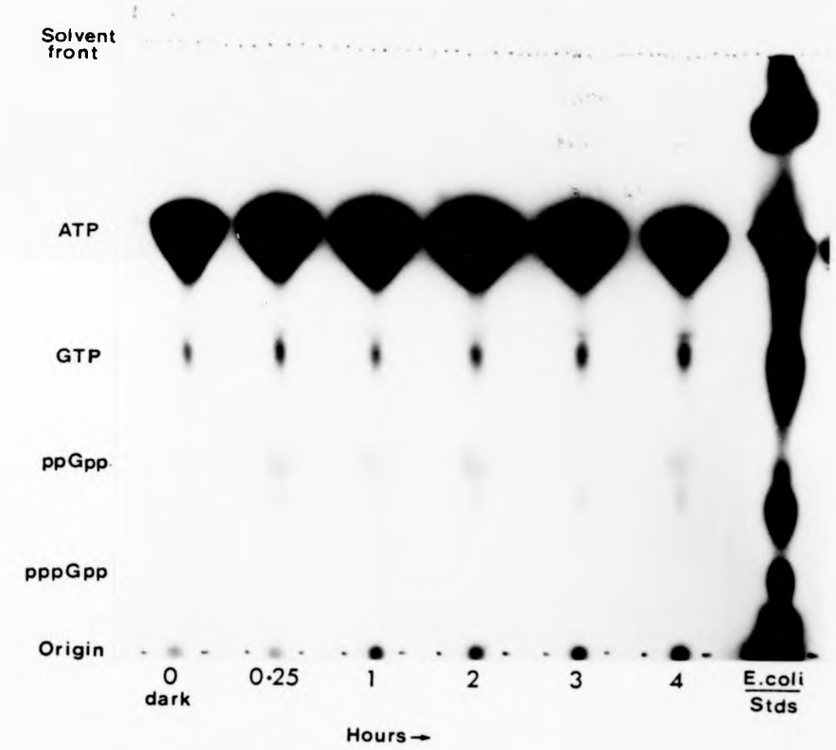


Figure 5.2.

Examination of Inhibited *R. vannielii* Swarmer Cells for the Presence of Highly Phosphorylated Nucleotides During Incubation in the Dark.

Batch cultures of *R. vannielii* were labelled throughout growth with 50 μCi ml^{-1} ^{32}P-orthophosphate and swarmer cells were selected during the late exponential phase of growth. The resulting cells were inhibited from initiating maturation by incubation in the dark and were sampled at the times indicated. Samples were prepared for thin layer chromatography and autoradiography as described in Chapter 2. This figure shows the resulting autoradiograph.

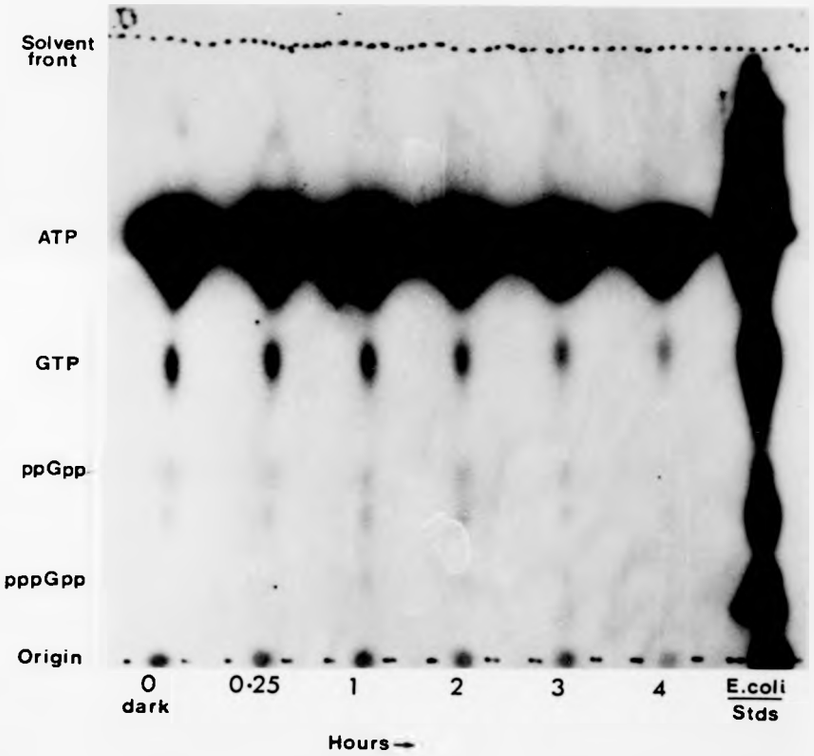


Figure 5.2.

Examination of Inhibited *R. vannielii* Swarmer Cells for the Presence of Highly Phosphorylated Nucleotides During Incubation in the Dark.

Batch cultures of *R. vannielii* were labelled throughout growth with 50 μCi ml^{-1} ^{32}P-orthophosphate and swarmer cells were selected during the late exponential phase of growth. The resulting cells were inhibited from initiating maturation by incubation in the dark and were sampled at the times indicated. Samples were prepared for thin layer chromatography and autoradiography as described in Chapter 2. This figure shows the resulting autoradiograph.

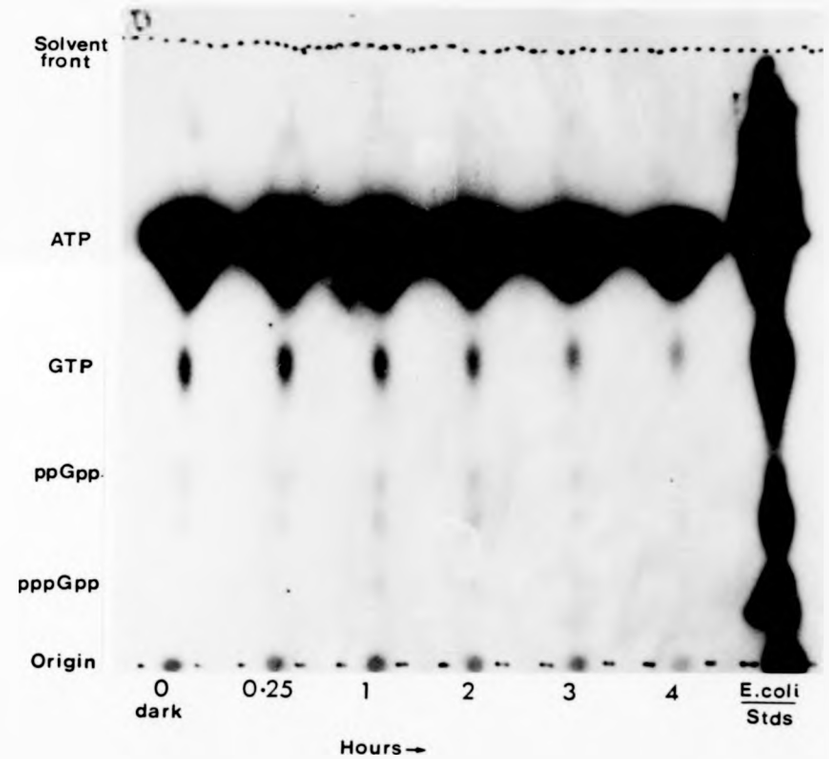


Figure 5.3.

Examination of *R. vannielii* Swarmer Cells for the Presence of Highly Phosphorylated Nucleotides During Differentiation and Subsequent Incubation in the Dark.

Batch cultures of *R. vannielii* were labelled throughout growth with 50 μCi ml^{-1} ^{32}P-orthophosphate and swarmer cells were selected during the late exponential phase of growth. The resulting cells were allowed to develop and sampled at the times indicated. Developing cultures were shifted to incubation in the dark immediately following the three hour sampling. Samples were prepared for thin layer chromatography and autoradiography as described in Chapter 2. This figure shows the resulting autoradiograph.

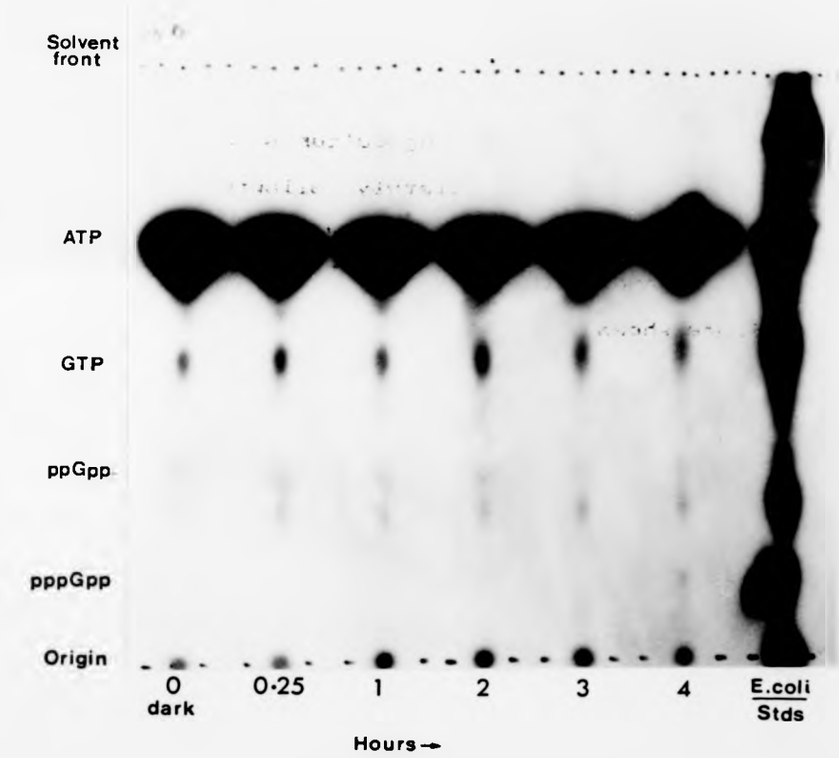
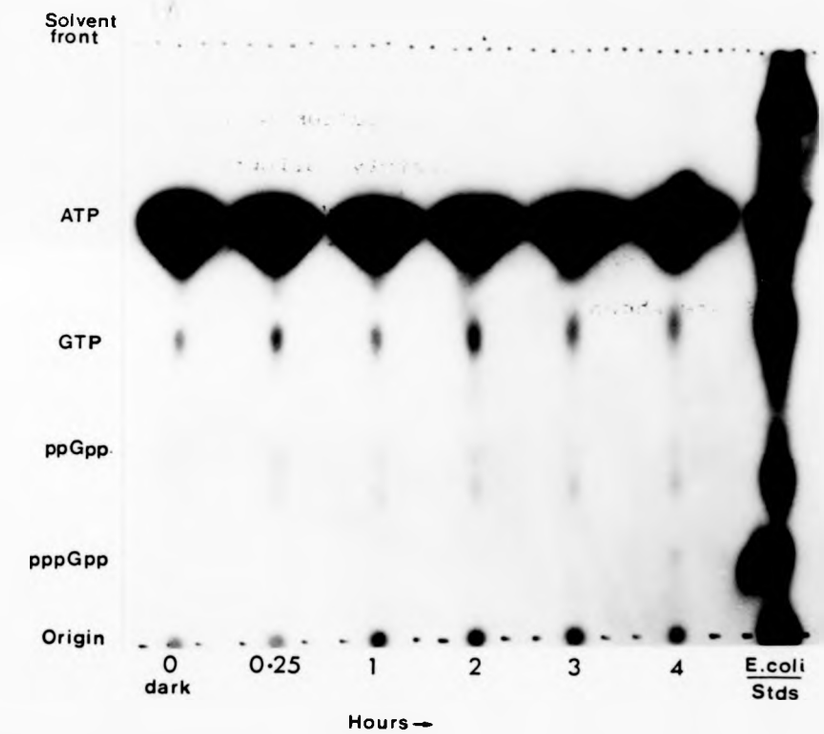


Figure 5.3.

Examination of *R. vannielii* Swarmer Cells for the Presence of Highly Phosphorylated Nucleotides During Differentiation and Subsequent Incubation in the Dark.

Batch cultures of *R. vannielii* were labelled throughout growth with 50 μCi ml^{-1} ^{32}P-orthophosphate and swarmer cells were selected during the late exponential phase of growth. The resulting cells were allowed to develop and sampled at the times indicated. Developing cultures were shifted to incubation in the dark immediately following the three hour sampling. Samples were prepared for thin layer chromatography and autoradiography as described in Chapter 2. This figure shows the resulting autoradiograph.



bacteria *Anacystis nidulans* (Mann *et al* 1975) and *Rhodo-pseudomonas sphaeroides* (Campbell & Lueking 1983) that a shift down in light causes a clear increase in the level of these HPN's. Likewise, when differentiating *R. vannielii* stalked cells were placed inthe dark a small increase in the level of pppGpp (but not ppGpp) was observed (figure 5.3), suggesting that in this case a stringent response was induced. It should be noted that the increase in the level of pppGpp was observed 1 hour after the shift into the dark. This may represent a decaying stringent response (see Mann *et al* 1975), though in *Rhodopseudomonas sphaeroides* the peak response occurs approx. one hour after a shift into the dark (Campbell & Lueking 1983).

5.2 Examination of *R. vannielii* rDNA for Evidence of DNA Rearrangements or Variations in rDNA Methylation Patterns During the Cell Cycle.

Introduction.

Genomic rearrangements and DNA methylation have also been examined as possible mechanisms by which rRNA synthesis could be regulated in *R. vannielii*. In general, both of these mechanisms mediate a less transient form of gene control than that afforded by the stringent response. However, the transition from the inhibited to the differentiating swarmer cell is accompanied by a number of permanent changes which are typified by the loss of the ability to synthesize flagella.

DNA rearrangements are invariably associated with repetitive sequences and it has been shown that *R. vannielii* has a large number of inverted repeat sequences (IRS) representing 7 % of the genome (Russell & Mann 1986). A similarly high level of IRS is also apparent in other differentiating organisms in contrast to *E. coli* that exhibits a monomorphic cell cycle and possesses less than 1 % IRS (Kato *et al* 1974). Needless to say, a role has been sought for the high levels of IRS within the process of differentiation. The only clear case in which a relationship has been shown is during heterocyst differentiation in the cyanobacterium *Anabaena* 7120 (Golden *et al* 1985), where an 11 kb segment of genomic DNA is excised to bring two nitrogenase structural genes *nifK* and *nifD* together. This rearrangement is presumably necessary for the functional expression of nitrogenase.

In eukaryotes amplifying DNA rearrangements are known to be involved in the expression of rDNA (Gall 1969). This phenomenon has not found in prokaryotes, but rearrangements have been documented between homologous rRNA genes in *E. coli* (Hill & Harnish 1981). The function, if any, of these recombinational events is unclear; growth rate is not significantly affected. In addition, prokaryotic rRNA genes are all flanked by nearly perfect IRS that historically have been associated solely with the processing of rRNA precursors (Abelson 1979). At this point it is tempting to speculate on an additional function for these IRS in genetic recombination.

In the eukaryotic world, differential DNA methylation is clearly implicated in gene regulation (Doerfer 1983) and in particular rRNA gene regulation (eg. Bird *et al* 1981a). In prokaryotes, information on the role of DNA methylation as a modulator of gene expression is limited and is reviewed in Chapter 1. Interestingly, Degnen and Morris (1973) report a two to three-fold higher level of DNA methylase activity in *Caulobacter bacteroides* stalked cells than is found in the swarmer cells. It seems likely, however, that this difference only reflects the need of the stalked cell to methylate the newly replicated DNA strands. However, it is important to stress that in eukaryotes gene regulation is generally determined by variations in DNA methylation at specific sites (Doerfler 1983) rather than by gross levels of methylation.

In essence, the approach to searching for cell cycle associated variations in DNA methylation in *R. vannielii* parallels that for studying DNA rearrangements. The salient point in this case, is that the restriction enzymes chosen, exhibit differential sensitivity to site specific methylation within their recognition sequences (Table 5.1).

5.2.1 Summary.

No evidence was found for the occurance of either rDNA rearrangements or changes in the pattern of rDNA methylation during the cell cycle of *R. vannielii*

5.2.2 Experimental Details.

R. vannielii DNA was isolated from inhibited swarmer cells, stalked cells and heterogeneous cell cultures. The DNA was restricted with a number of endonucleases, fractionated by agarose gel electrophoresis, Southern blotted onto nitrocellulose and probed with end-labelled total *R. vannielii* rRNA.

Variations in autoradiograph banding patterns may have indicated a change in the structure of the DNA by physical rearrangement. Alternatively, the restriction endonucleases chosen all show a degree of sensitivity to methylation of specific bases within their recognition sequences. These specificities are summarized in table 5.1. The differential sensitivity of the isochizomers *Msp*I and *Hpa*II have been particularly instrumental in elucidating the role of DNA methylation in eukaryotic gene control.

The results are shown in figure 5.4 and 5.5. No qualitative changes in banding patterns were observed. No evidence for either rDNA rearrangements or changes in the pattern of rDNA methylation during the cell cycle of *R. vannielii* was found. It should, however, be borne in mind that the method used would not detect rearrangements that occurred within the limits of a particular restriction fragment, or changes in DNA methylation that did not occur within the recognition sites of the restriction endonucleases used. However, the use of *Msp*I and *Hpa*II that both recognize and restrict tetranucleotide sequences

facilitated relatively fine examination.

Figure 5.4.

Examination of *R. vannielii* rDNA Sequences for Rearrangements and Site Specific Variations in Methylation During Swarmer Cell Maturation.

DNA's from *R. vannielii* swarmer cells, stalked cells and heterogeneous cell populations were restricted as indicated. These digests were fractionated by agarose gel electrophoresis, Southern blotted onto a nitrocellulose filter and probed with total *R. vannielii* rRNA at 95 % stringency.

Key:

A Heterogeneous DNA restricted with *Eco*R1

B Heterogeneous DNA restricted with *Hind*III

C Swarmer cell DNA restricted with *Eco*R1

D Swarmer cell DNA restricted with *Hind*III

E Stalked cell DNA restricted with *Eco*R1

F Stalked cell DNA restricted with *Hind*III

G Autoradiograph of A probed with total *R. vannielii* rRNA

H Autoradiograph of B probed with total *R. vannielii* rRNA

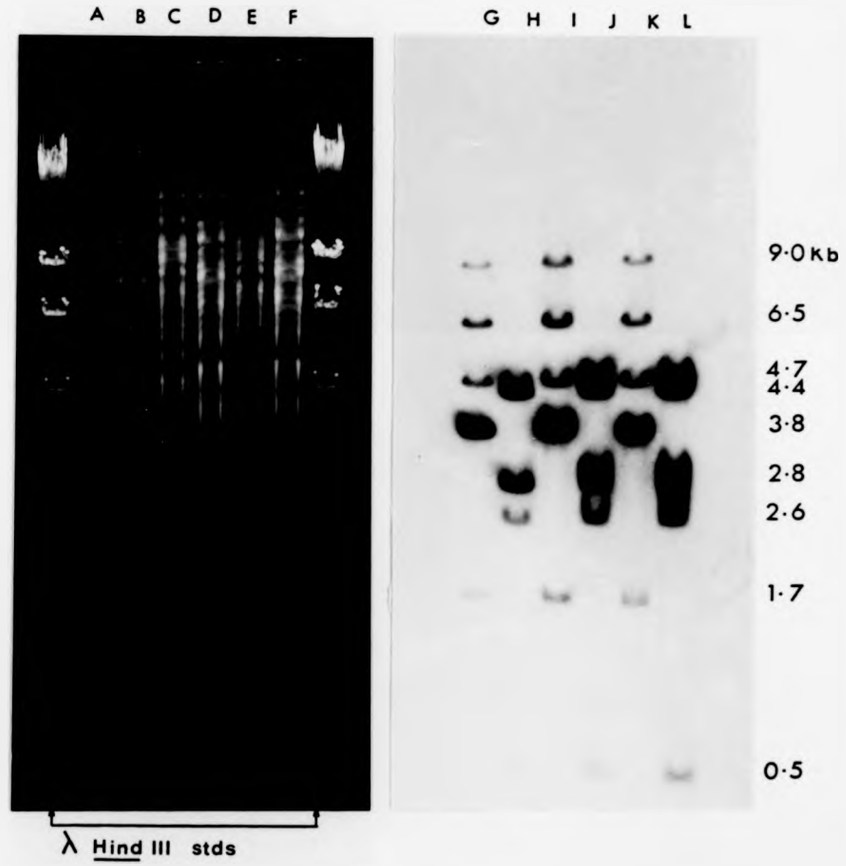
I Autoradiograph of C probed with total *R. vannielii* rRNA

J Autoradiograph of D probed with total *R. vannielii* rRNA

K Autoradiograph of E probed with total *R. vannielii* rRNA

L Autoradiograph of F probed with total *R. vannielii* rRNA

(Refer to Table 5.1 for *Eco*R1 and *Hind* III functional specificities).



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Key:

A Heterogeneous DNA restricted with *Eco*R1

B Heterogeneous DNA restricted with *Hind*III

C Swarmer cell DNA restricted with *Eco*R1

D Swarmer cell DNA restricted with *Hind*III

E Stalked cell DNA restricted with *Eco*R1

F Stalked cell DNA restricted with *Hind*III

G Autoradiograph of A probed with total *R. vannielii* rRNA

H Autoradiograph of B probed with total *R. vannielii* rRNA

I Autoradiograph of C probed with total *R. vannielii* rRNA

J Autoradiograph of D probed with total *R. vannielii* rRNA

K Autoradiograph of E probed with total *R. vannielii* rRNA

L Autoradiograph of F probed with total *R. vannielii* rRNA

(Refer to Table 5.1 for *Eco*R1 and *Hind* III functional specificities).

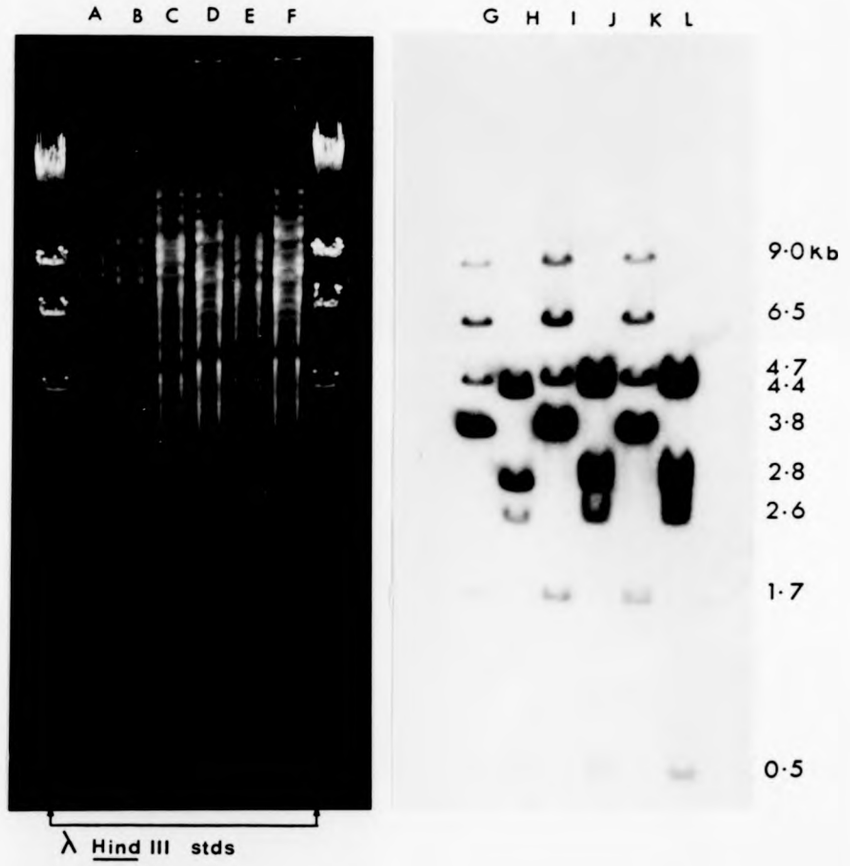

Figure 5.5.

Examination of *R. vannielii* rDNA Sequences for Rearrangements and Site Specific Variations in Methylation During Swarmer Cell Maturation.

DNA's from *R. vannielii* swarmer cells and stalked cells were restricted as indicated. These digests were fractionated by 5 % (w/v) polyacrylamide gel electrophoresis, Southern blotted onto a nitrocellulose filter and probed with total *R. vannielii* rRNA at 95 % stringency.

Key:

A Stalked cell DNA restricted with *Msp*I

B Swarmer cell DNA restricted with *Msp*I

C Stalked cell DNA restricted with *Hpa*II

D Swarmer cell DNA restricted with *Hpa*II

E Autoradiograph of A probed with total *R. vannielii* rRNA

F Autoradiograph of B probed with total *R. vannielii* rRNA

G Autoradiograph of C probed with total *R. vannielii* rRNA

H Autoradiograph of D probed with total *R. vennielii* rRNA

(Refer to Table 5.1 for *Msp*I and *Hpa*II functional specificities).

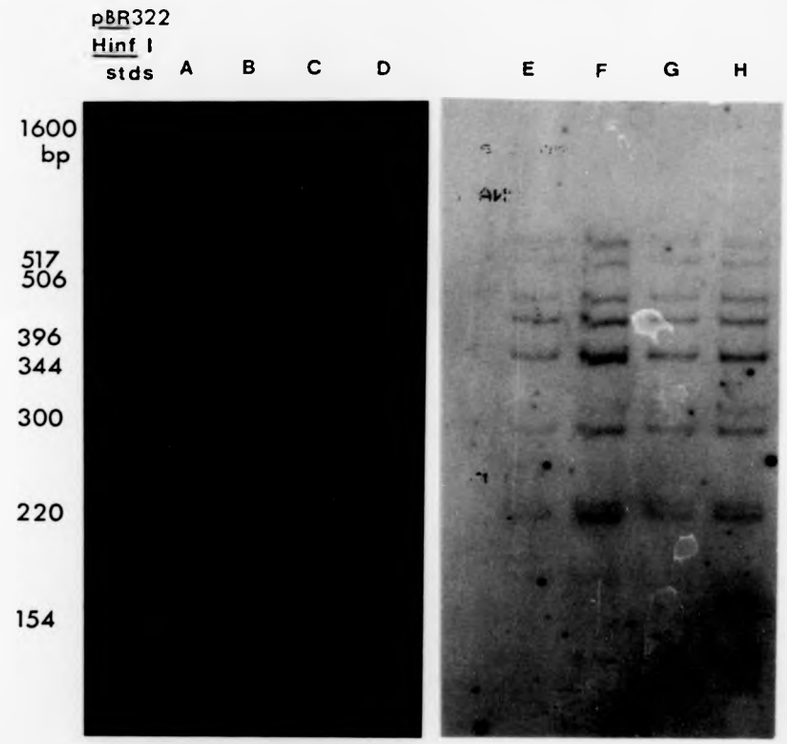

Figure 5.5.

Examination of *R. vannielii* rDNA Sequences for Rearrangements and Site Specific Variations in Methylation During Swarmer Cell Maturation.

DNA's from *R. vannielii* swarmer cells and stalked cells were restricted as indicated. These digests were fractionated by 5 % (w/v) polyacrylamide gel electrophoresis, Southern blotted onto a nitrocellulose filter and probed with total *R. vannielii* rRNA at 95 % stringency.

Key:

A Stalked cell DNA restricted with *Msp*I

B Swarmer cell DNA restricted with *Msp*I

C Stalked cell DNA restricted with *Hpa*II

D Swarmer cell DNA restricted with *Hpa*II

E Autoradiograph of A probed with total *R. vannielii* rRNA

F Autoradiograph of B probed with total *R. vannielii* rRNA

G Autoradiograph of C probed with total *R. vannielii* rRNA

H Autoradiograph of D probed with total *R. vennielii* rRNA

(Refer to Table 5.1 for *Msp*I and *Hpa*II functional specificities).

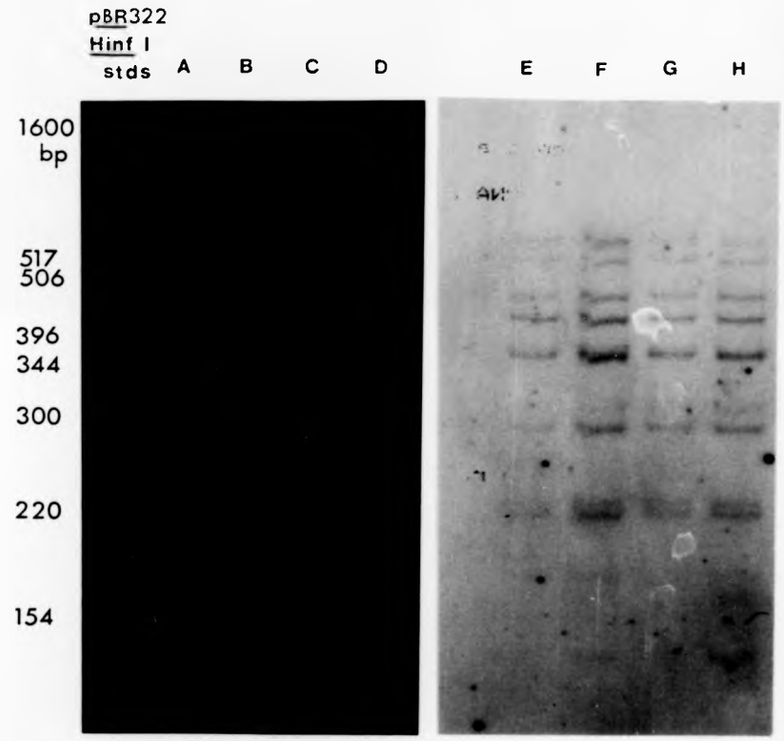

Table 5.1.

Specificity of Restriction Endonucleases With Respect to Site Specific DNA Methylation.

Restriction enconuclease		CCGG	^{m}CCGG	^{m}CCGG	
*Hpa*II	(1,2)	+	+	−	
*Msp*I		+	−	+	
		GAATTC	^{m}GAATTC	^{m}GAATTC	^{m}GAATTC
*Eco*R1	(3)	+	+	−	−
		AAGCTT	^{m}AAGCTT		
*Hind*III	(3)	+	−		

Key:

\+ restricted

− not restricted

Refs:

1 Waalwijk & Flavell (1978)

2 Sneider (1980)

3 McClelland & Nelson (1985)

Chapter 6

Conclusions

Conclusion.

The regulation of ribosome synthesis in the prokaryotes has been studied extensively in *E. coli* but comparatively little in other organisms. However, the simple characteristics of this monomorphic organism are by no means typical of prokaryotes in general. For this reason it was of interest to examine this subject in the polymorphic organism *R. vannielii* and particularly in the the non-growing, but active, dark-inhibited swarmer cell.

It is clear that the dark-inhibited swarmer cell needs to conserve energy, with the obvious exception of its active motility, which may be considered as being obligatory to this cells role in dispersal (Dow *et al* 1983). One might expect, however, that all other unnecessary and energy wasting processes would be arrested, as are those cellular functions associated with growth and maturation. The synthesis of the translational apparatus represents a massive energy drain upon a cell and is therefore a prime candidate to be arrested and possibly by an alternative mechanism to those previously characterized for *E. coli*.

In *R. vannielii*, rRNA synthesis was shown to occur throughout the process of swarmer cell maturation and reproduction. However, it was not possible to conclusively elucidate the situation for the dark-inhibited swarmer cell which failed to incorporate any of the several nucleotide precursors tested. It is possible that this result simply reflects this cells inactivity in both DNA replication and

rRNA synthesis . However, it is also possibile that in this cell, nucleotide uptake is inhibited. It should be noted that for all labelling experiments described in this thesis, TCA-precipitable counts were measured i.e. a measure of incorporation and not uptake. Pulse-labelling of cells followed by washing in fresh medium prior to scintillation counting could be carried out to address the question of uptake.

Two *R. vannielii* rDNA clones, pCO1 and pCO2, were isolated to facilitate further studies on the regulation of ribosome synthesis in this organism and indeed pCO1 is at present being used to address this problem (Swoboda, personal communication). Synchronized, inhibited and differentiating swarmer cells can be made permeable by toluene treatment as described by Scott and Dow (JGM in press). These permeable cells will be pulse-labelled with ^{32}P-adenosine triphosphate and any resulting rRNA transcripts will be detected by hybridization to the 16S rRNA coding region of pCO1. This approach has the advantage in avoiding possible variations in membrane permeability. No results are available at this point in time.

Recent advances in the areas of mutagenesis and gene transfer for *R. vannielii* (see chapter 1) will doubtless have a very significant effect upon cell cycle research in this organism. These techniques will allow the construction, manipulation and characterization of a variety of cell cycle mutations. They will also be of great importance in studying the control of ribosome synthesis in *R. vannielii*. Ribosomal

promoters can be fused to marker genes with easily identifiable products allowing a greatly simplified assay of promoter activity. Should such an assay prove successful, putative regulatory sequences can be examined by an 'alter and assay' approach.

It was shown that ^{35}S-methionine is incorporated by dark-inhibited swarmer cells at a rate of approximately 15 % of that seen for differentiating swarmer cells. It has therefore been possible to use ^{35}S-methionine labelling to show that r-protein synthesis does occur in dark-inhibited swarmer cells. That r-protein synthesis occurs in a cell that exhibits no increase in its overall mass, indicates that this cell must also turnover its ribosomes. At this point it is worth noting that Russell & Mann (1984) have previously shown the presence of several serine hydrolases in the dark-inhibited swarmer cell.

It would appear to be very wasteful for a non-growing, energy conserving cell to use up valuable energy resorces to make a product that is simply destined to be broken down again. However, it has been argued that a cell can more readily increase a previously low level of synthesis than it can start up a new one (Nomura *et al* 1984). Perhaps by maintaining a low level of ribosome synthesis, the dark inhibited swarmer cell is able to respond more rapidly, in terms of growth, to a favourable environment.

The method used to examine r-protein synthesis in this thesis was significantly limited in both its requirement

for a large cell number (to facilitate ribosome isolation) and its lack of sensitivity. In order to examine the regulation of r-protein synthesis more closely, a more sensitive method is required. This might be achieved by using immunoprecipitation to facilitate the purification of *R.vannielii* r-proteins. Such an approach should allow pulse-labelling of small culture volumes with high levels of ^{35}S-methionine. If successful, pulse chase experiments could be employed to examine the turnover of r-protein.

In several organisms that have previously been examined, a shift down in energy is accompanied by an increase in the level of ppGpp which in turn is taken to lead to a decrease in the rate of ribosome synthesis. The level of ppGpp was examined in *R. vanneilii* but was found to be no higher in the dark inhibited swarmer cell than in their maturing counterparts. It seems therefore, that the physiological state of the dark-inhibited swarmer is not due to a stringent responce or a growth rate regulation via ppGpp.

Two mechanisms by which rRNA synthesis has been shown to be regulated in eukaryotes were examined, i.e. specific methylation of rDNA and rearrangements/amplification of rDNA sequences. No evidence for the action of either mechanism was obtained, though the techniques used can not absolutely rule out the possibility of either functioning.

The final line of research in this thesis examined the organization of the rRNA genes in *R. vannielii*. This work involved mapping rRNA sequences on restriction endonuclease

digests of genomic DNA and the cloning of two DNA fragments that contained 16S rRNA sequences. The former approach indicated the presence of at least two conventional eubacterial rRNA operons in *R.vannielii*. However, two further bands were detected that exhibited abnormally weak signals with both 23S and 16S rRNA probes. Further evidence for an atypical eubacterial rDNA organization comes from the two *R. vannielii* ribosomal clones pCO1 and pCO2 each of which appears to contain a single unlinked 16S rRNA sequence. Unfortunately, the relevance of these atypical sequences is unclear and it remains possible that they represent pseudogenes that no longer have a function in ribosome synthesis. It would be of great interest to further characterize the rDNA of *R. vannielii* by further mapping, cloning and expressional studies.

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