STRUCTURE OF THE R.VANNIELII GENOME

by

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to MUM and DAD

SUMMARY

The control of cellular morphogenesis and differentiation at the molecular level in Rhodomicrobium vannielii is investigated by biochemical and genetic analysis. Chemical characterization indicates that the R.vannielii genome is 2.19×10^9 daltons, and is made up of 95 per cent unique DNA sequence.

Five per cent of the DNA consists of short inverted repeat sequences, 400 bp in length. No extrachromosomal DNA is detectable. The DNA from each of the cellular expressions in R.vannielii does not show any major differences in sequence composition.

Kinetic analysis of nucleic acid synthesis during the obligate differentiation of the swarm cell shows a 'lag' or maturation period prior to the onset of DNA replication, whereas no lag occurs in RNA synthesis. The initiation of DNA replication during swarm cell differentiation occurs towards the completion of stalk synthesis.

Studies with the protein synthesis inhibitor chloramphenicol during swarm cell differentiation demonstrate a requirement for protein synthesis in the initiation, but not the elongation step of DNA replication. Nalidixic acid inhibits DNA synthesis in the swarm cell, and although a new daughter cell is produced, cell division does not occur. This implies that chromosome replication and cell division are directly linked in a 'dependent pathway' of events. Daughter cell synthesis is under the control of the mother cell genome, the daughter cell genome becoming metabolically active just prior to cell division. Further 'cells' produced in nalidixic acid-treated cultures show gross cellular distortion and no stalk formation.

The development of a genetic system for R.vannielii is discussed. The promiscuous plasmid R.68.45 is transferred by conjugation from E.coli to R.vannielii, where it expresses only one of three plasmid-borne antibiotic resistances, but may be maintained intact in the bacterium. This plasmid may now be used in mapping the R.vannielii chromosome.

Analysis of the R.vannielii genome by restriction enzyme cleavage is described. Restriction fragments containing the coding sequences for 16s rRNA and 23s rRNA are identified by 'Southern' hybridization, and their sizes estimated. Attempts to locate the origin of replication on a single restriction fragment are discussed. Preliminary data on the physiology of nitrogen fixation in R.vannielii shows that the enzyme system is inducible.

The potential of this organism as a model system for further study on the molecular biology of microbial morphogenesis and differentiation is discussed.

DECLARATION

I hereby declare that this thesis is the results of original research conducted by myself, under the supervision of Dr. C. S. Dow. The renaturation experiments were done in collaboration with Dr. R. J. Avery, and the electron microscopy of DNA in collaboration with Dr. J. H. Parish (University of Leeds).

Linda E Potts

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I wish to acknowledge the help of Dr. Roger Avery with the thermal renaturation experiments, and of Dr. Howard Parish with the electron microscopy of DNA.

I would like to thank all members of the Micro squad particularly Uthaya Swoboda, John Colby, Dave Stirling, Kevin Eckersley and Paul Taylor for generating a happy atmosphere in the lab, and for entertaining discussions on all subjects. Also, thanks to many other members of Biological Sciences for help, advice and discussions.

Many thanks are due to Dr. Howard Dalton and Dr. Tony Bradshaw for their reading of the thesis, and to Anita Davies for her fast and accurate typing.

Finally, thanks to Tony for his continued support and encouragement.

ABBREVIATIONS

A_{540nm}	Absorbance at 540nm
A.T.P.	adenosine-5'-triphosphate
bp	base pair
B.S.A.	bovine serum albumin
chl	chloramphenicol
Cot	concentration of DNA x time
ccc	covalently closed circle
DNA	deoxyribonucleic acid
dATP	3'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	thymidine-5'-triphosphate
dTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
kbp	kilobase pair
λ	lambda
nal	nalidixic acid
NEM	n-ethylmaleimide
nif	nitrogen fixation
NTG	N-methyl-n'-nitro-N-nitrosoguanidine
pCMS	p-chloromethylphenylsulphate
POPOP	1,4-bis-(5-phenyloxazole-2-yl) benzene
PPO	2,5 diphenyloxazole
RNA	ribonucleic acid

rRNA	ribosomal RNA
mRNA	messenger RNA
tRNA	transfer RNA
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
TCA	trichloroacetic acid
TRIS	tris(hydroxymethyl)aminomethane.
U.V.	ultra violet
v/v	volume to volume
w/v	weight to volume

CONTENTS

CHAPTER 1

Introduction

1.1. General introduction and definition of terms

The nature of the regulation of cellular morphogenesis and differentiation is a major unanswered question in biology. During the past ten years, the area has been studied extensively in both prokaryotic and eukaryotic systems (Piggott and Coote, 1976; Doi, 1977a; O'Malley et al., 1977), and although a vast literature has accumulated, the basic regulatory processes remain enigmatic.

In eukaryotes, development is a composite process involving cellular morphogenesis and differentiation with subsequent cellular interactions in tissues or organs. The mechanisms underlying such a complex process are difficult to delineate experimentally. Consequently considerable effort is being directed towards an understanding of the processes involved in morphogenesis and differentiation in the less complex prokaryotic systems, not only for their own sake, but also in the belief that such knowledge will prove valuable in elucidating the regulatory mechanisms in the eukaryote. Recent work has demonstrated however, that the molecular biology of higher organisms is markedly different from that of prokaryotes.

In eukaryotes, in contrast to prokaryotes, large-scale gene amplification is found (Brown and Gurdon, 1964), and many genes contain intervening sequences within the coding regions (Breathnach et al., 1977). These 'introns' are transcribed, necessitating a processing event by an (as yet) unknown mechanism, which has been termed 'splicing' (Crick, 1979), to generate the mature mRNA. No prokaryotic genes have been shown to contain intervening sequences. Nonetheless, prokaryotic differentiation is an exciting field in its own right, encompassing the areas of differential gene expression, transcriptional and translational control, and post-translational processing.

The aim of this introduction is to illustrate the diversity of prokaryotic differentiation. Particular emphasis is placed on certain selected organisms in an effort to review the current understanding of the regulatory processes involved in the expression of simple differentiation events in the prokaryotic cell cycle.

It is necessary, before considering the prokaryotes which have been used in the study of differentiation, to define several terms, the meanings of which are often confused.

a) Morphogenesis refers to changes in the internal architecture and external morphology of the cell during the cell cycle.

b) Differentiation refers to a series of events which are initiated by a 'switch' in the cell cycle, leading to the formation of a different cell type. Differentiation may be reversible or permanent.

c) Development is a composite event involving morphogenesis and differentiation under intercellular influence. For example, in some cyanobacteria certain cells (the heterocysts) are modified to perform a particular function necessary to the activity of the multicellular complex.

d) 'Monomorphic vegetative cell cycle type'prokaryotes are those in which there is only one morphological growth form under normal nutrient conditions, and which always exist in the vegetative phase e.g. Escherichia coli.

e) 'Dimorphic cell cycle type' prokaryotes are those which at division, produce two cell types which differ from each other in size and/or shape. The transition between these two is an obligate, irreversible part of the cell cycle, e.g. Caulobacter crescentus.

f) 'Polymorphic cell cycle type' prokaryotes are those which show two or more physiologically distinct types of cell, each of which undergoes a distinctive and constant cell cycle. Different morphological forms may be induced by changes in nutrient conditions e.g. Arthrobacter, Geodermatophilus.

1.2. Morphogenesis and differentiation in prokaryotes - an overview

Many prokaryotes have been used as model systems for the analysis of cellular differentiation (Table 1.1). Early studies of differentiating systems focussed on the gross morphological manifestations of the process, which were observed by light microscopy, and more recently by electron microscopy (Vatter et al., 1959; Conti and Hirsch, 1965). In the past ten to fifteen years techniques developed in molecular biology have been increasingly applied to studies of cellular morphogenesis and differentiation, with considerable success.

Morphogenesis in Geodermatophilus was studied by examining the effect of nutrients on the induction of different cell shapes. Ishiguro and Wolfe (1970) showed that a factor present in tryptose is responsible for the induction of differentiation (Fig. 1.1), and later showed that mono and divalent cations induce the conversion of budding rods to cocci, and maintain the coccoid form (Ishiguro and Wolfe, 1974).

Arthrobacter crystallopoietes exhibits a nutritionally induced differentiation from sphere to rod, which occurs within a completely vegetative cell cycle (Fig. 1.2; Ensign and Wolfe, 1964). The transition may be controlled by manipulation of the growth medium, but the causative agent remains unknown. The major limitation to a comprehensive study of both Arthrobacter and Geodermatophilus is that

Table 1.1. Prokaryotes which have been used for studies of cellular morphogenesis and differentiation

Organism	Reference
Anabaena cylindrica	Adams and Carr (1979)
Arthrobacter	Ensign and Wolfe (1964)
Bacillus subtilis	Doi (1977b)
Caulobacter crescentus	Shapiro (1972)
Chlorogloea fritschii	Evans *et al.*, (1976)
Geodermatophilus	Ishiguro and Wolfe (1970)
Hyphomicrobium	Hirsch (1974)
Myxococcus	Wireman and Dworkin (1977)
Rhodopseudomonas palustris	Westmacott and Primrose (1976)
Rhodomicrobium vannielii	Whittenbury and Dow (1977)

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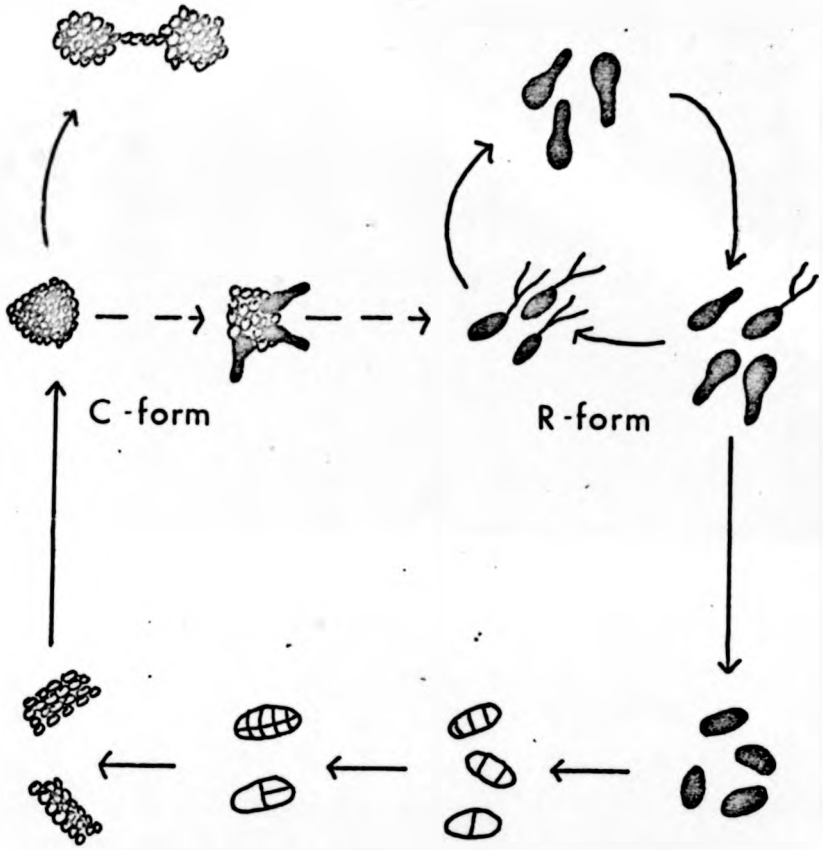


Fig. 1.1. The cell cycle of Geodermatophilus strain 22-68*

Growth and cell division in the C-form requires the presence of a factor found in tryptose. Absence of this factor induces differentiation to the R-form. Re-addition of tryptose will induce differentiation from the R-form to the C-form. Later data (Ishiguro and Wolfe, 1974) indicated that mono and divalent cations induced the conversion of budding rods to cocci.

* From Ishiguro and Wolfe (1970).

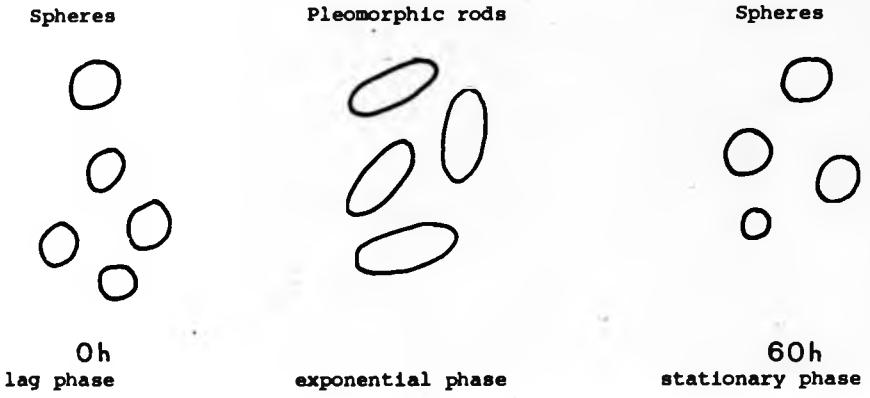


Fig. 1.2. The morphogenetic cell cycle of Arthrobacter

The morphogenetic transition occurs in one complete vegetative cell cycle. Growth is entirely in the coccoid form when cells are grown on glucose and minimal salts. Addition of L-arginine or any of a range of amino acids induces rod formation which is maintained until the 'inducer' is exhausted.

the genetic and biochemical knowledge of them is slight.

Both unicellular and multicellular blue-green algae have been exploited in studies of cellular differentiation. The unicellular Chlorogloea fritschii shows a wide range of phenotypic variations in morphology as a result of different environmental stimuli (Fig. 1.3; Evans et al., 1972). The complexity of this system makes analysis of the underlying molecular events extremely difficult.

In the filamentous blue-green alga Anabaena cylindrica, the formation of heterocysts (cells specialized for nitrogen fixation) follows a pattern determined by the surrounding vegetative cells (Wilcox et al., 1973a). It was suggested that individual heterocysts produce an inhibitor of further heterocyst development, so determining that new heterocysts may only develop at a certain distance further down the filament, where the concentration of inhibitor is below a critical threshold level. Breakage of the filament near a proheterocyst causes it to regress to a vegetative cell, since the breakage will allow the accumulation of inhibitor in the proheterocyst (Wilcox et al., 1973b).

Treatment of A.cylindrica with specific metabolic inhibitors at various times after transfer to ammonia-free medium showed that proheterocyst development occurs in the minimum concentrations of inhibitors which prevent mature heterocyst formation (Adams and Carr, 1979). The inhibitory effect of mitomycin C is mediated earlier in the developmental sequence than that of rifampicin. The effects of the two inhibitors on proheterocyst and heterocyst development are different but parallel, suggesting that at least two separate genes, one for proheterocyst, and one for mature heterocyst formation, are operating.

Although studies on the molecular biology and biochemistry of A.cylindrica are progressing, in general the other systems described above suffer from the lack of an adequate biochemical and genetical background, and the patterns of differentiation are inherently extremely complex. Studies of several other organisms have been more extensive than those described above, and these will be discussed in detail. The rapid progress of research on these other prokaryotes was largely due to the biochemical knowledge already in existence. E.coli was the first bacterium in which regulatory mechanisms were examined in detail.

1.3. A simple monomorphic cell cycle - Escherichia coli

1.3.1. Growth and cell division. In studies concerned with the regulation of the cell cycle, E.coli would be the prokaryote of choice, because of the wide knowledge, accrued over the last twenty years, of the biochemistry and genetics of this organism. Moreover, its rapid growth rate, and the availability of easy culture techniques, make it an ideal experimental organism. In terms of cellular morphogenesis and differentiation, it possesses a simple morphogenetic cell cycle, in which cell division is the only visible morphological event. Cell division during the cell cycle is however, an integral part of cellular morphogenesis and differentiation, and E.coli has been used as a model system to investigate the regulation of cell division at the molecular level.

E.coli shows intercalary growth when growing rapidly in a complex medium (its generation time is less than 40 minutes), but when growing slowly, growth is polar, that is from one pole only (Fig. 1.4; Donachie et al., 1973). This polar growth is the same as the obligate polar growth shown by the budding bacteria (Whittenbury and Dow, 1977),

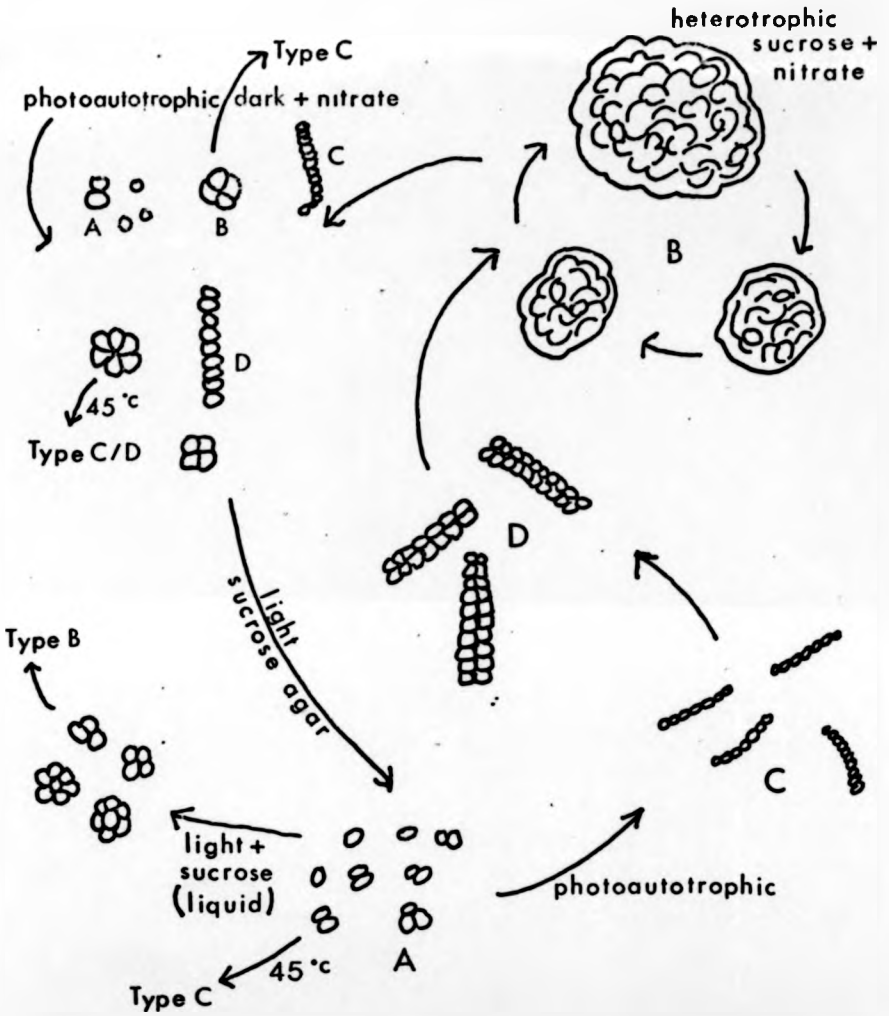


Fig. 1.3. Morphological variations induced in Chlorogloea fritschii by different environmental conditions*

Type A: large granulated cells (2 x 3 μm) existing either singly, or as clumps containing 2 or 3 cells which arise from division in 1-3 planes.

Type B: found in clumps which combine larger groups of cells surrounded by a mucilaginous sheath.

Type C: small cells (1 μm) found in filaments.

Type D: cells (1.5 μm) found in filaments in the process of dividing.

* From Evans et al., (1976)

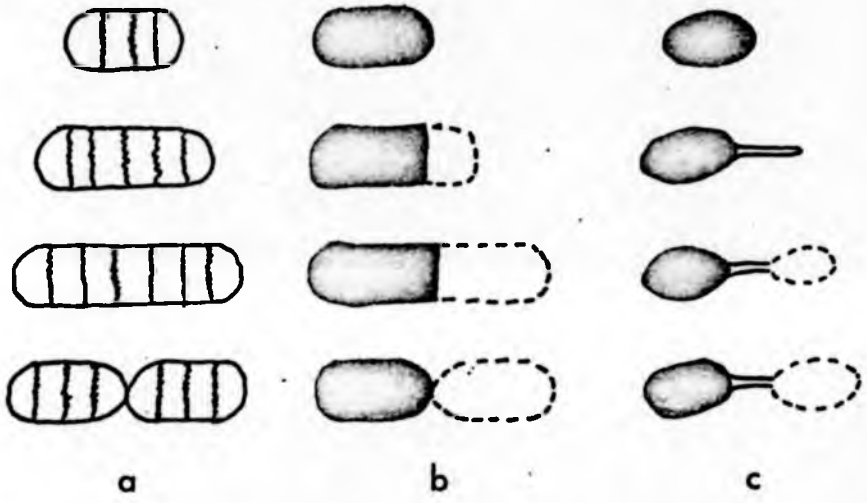


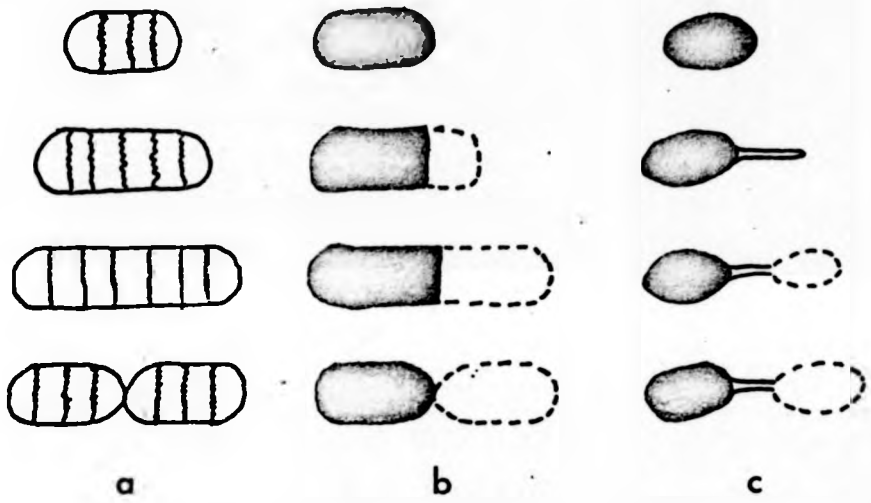
Fig. 1.4. Bacterial growth modes*

a) Multiple growth points characteristic of E.coli growing with a generation time of less than 40 min.

b) Polar growth shown by E.coli with a generation time in excess of 60 min (only one active growth point).

c) Obligate polar growth of a budding bacterium, e.g. Rhodomicrobium vannielii.

*. From Whittenbury and Dow (1977)



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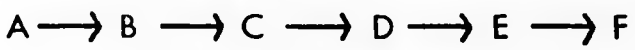
and cell division in both instances is primarily by binary fission, plug formation in Rhodomicrobium vannielii being the exception.

1.3.2. Relationship between cell division and chromosome replication. Early studies were directed towards elucidating the timing of DNA replication and cell division in the cell cycle of synchronous E.coli cultures, and determining the extent to which these two processes are coupled in the cell cycle. It was shown that in E.coli, termination of chromosome replication is a necessary pre-requisite for cell division (Clark, 1968; Helmstetter and Pierucci, 1968). Therefore when DNA replication is inhibited in complex medium, cell division does not occur.

However, chromosome replication is unlikely to be responsible for the precise timing of cell division (Jones and Donachie, 1973), and the temporal relationship between cell division and DNA replication has been shown not strictly to apply to individual E.coli cells (Kubitschek and Newman, 1978), where DNA replication is the most tightly controlled event. The rate of DNA replication has no effect on cell shape, and the lack of uniform shape demonstrated by thymine-starved cells is due to the imbalance of wall components, rather than the absence of DNA synthesis (Zaritsky and Woldringh, 1978).

1.3.3. The'dependent pathways'concept. Mitchison (1972) suggested two possible ways in which the sequence of cell cycle events may be fixed relative to one another. In the 'dependent pathway' model there is a direct causal connection between each event, so that completion of an earlier event is necessary before the next can occur. The'independent pathways' model envisages a series of parallel pathways operating under the control of a'master timer' (Fig. 1.5; Hereford and Hartwell, 1973).

a) 'Dependent pathway' model



b) 'Independent pathways' model

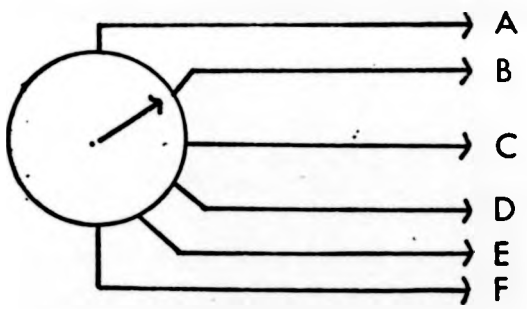


Fig. 1.5. Two models to account for the ordering of cell cycle events

In a), each event is dependent upon the completion of the previous event, whereas in b), there is a 'master clock' controlling several pathways of events, and each pathway is independent of the others.

The use of temperature-sensitive, cell division cycle (cdc) mutants has shown that in Saccharomyces cerevisiae, parallel control pathways are the most likely (Hartwell et al., 1974).

Jones and Donachie (1973) also proposed a model for E.coli in which DNA replication and the synthesis of 'division proteins' are in concurrent parallel pathways. The actual mechanism is probably one in which several parallel pathways are linked together by an over-riding mechanism, and within each individual pathway each event is dependent upon the completion of the previous one. This would result in a fine degree of control over cellular morphogenesis and differentiation.

1.3.4. The control of initiation of chromosome replication. Protein synthesis is a prerequisite for the initiation of DNA replication (Lark et al., 1963), but recently protein synthesis has also been implicated in chromosome termination (Jones and Donachie, 1973), and in parallel control pathways as indicated above. Jones and Donachie found protein synthesis requirements at the point of initiation of chromosome replication, during DNA replication and also in a period immediately after chromosome termination (Fig. 1.6). These three periods of protein synthesis show varying sensitivities to chloramphenicol. A contradictory report (Marunouchi and Messer, 1973) suggested that protein synthesis is required for replication of a terminal segment of the chromosome, and that replication of this segment is necessary for cell division. However, Loehr and Hanawalt (1977) could not confirm the existence of a unique terminal segment. These workers attributed the burst in incorporation of radioisotope seen in the earlier study, to a transient increase in the rate of replication fork movement induced by the temperature shift, rather than synthesis of a terminal

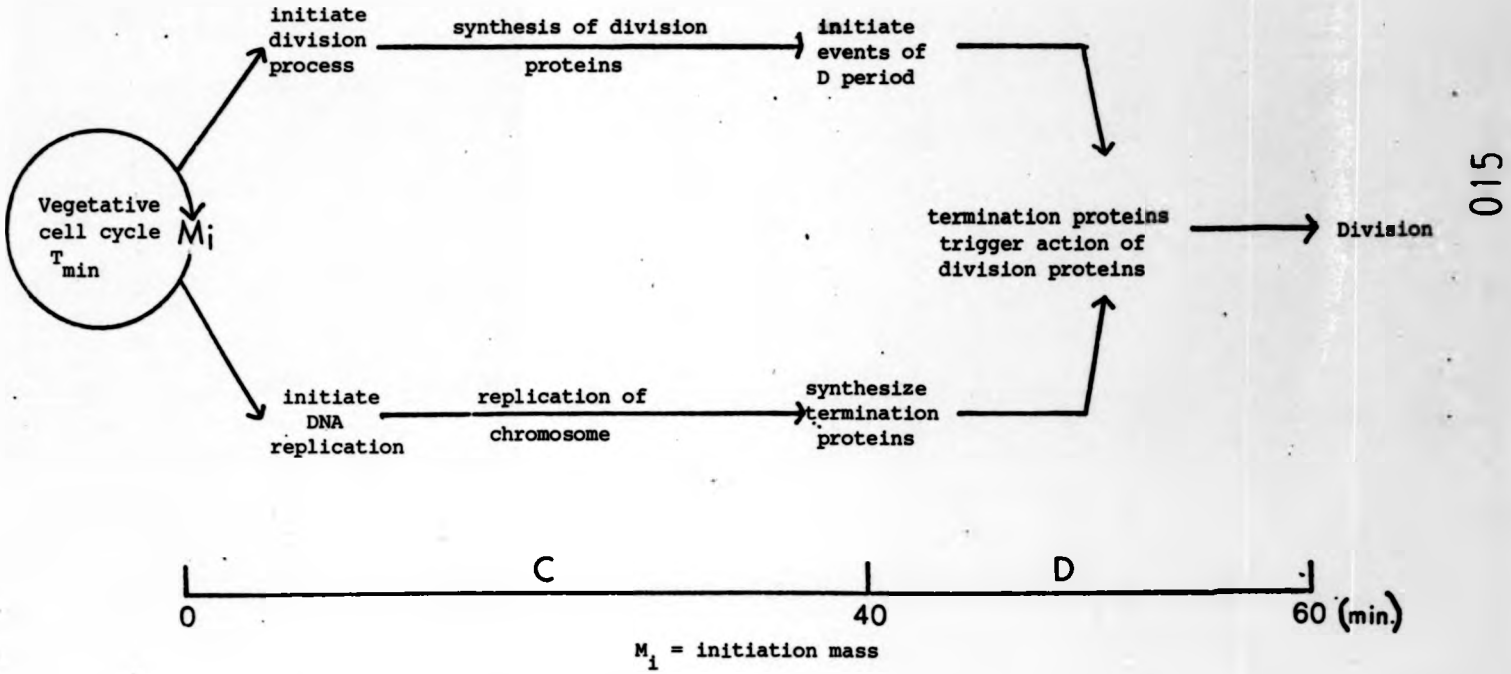


Fig. 1.6. Model of the cell cycle in Escherichia coli

part of the chromosome in response to the relieving of amino-acid starvation.

The initiation of DNA replication may be controlled in a positive or a negative manner. Jacob et al., (1963) suggested a positive mechanism, whereby an initiation protein would accumulate to a threshold level before triggering the onset of replication. The negative control mechanism supposes that an inhibitor or repressor is present in the cell, and replication only occurs when it is diluted below a threshold level by an increase in cell volume (Pritchard et al., 1969). This latter model is substantiated by the observation that some dnaA mutants show stimulation of initiation immediately after protein synthesis is inhibited (Tippe-Schindler et al., 1979).

Messer (1972) found that rifampicin inhibited initiation of replication at a time when inhibition of translation was ineffective. The fact that transcribing RNA polymerase is unaffected by rifampicin, allowed the size of the RNA species required for initiation to be estimated as 1000 nucleotides. RNA polymerase activity is implicated directly by the finding that the conversion of single stranded (SS) M13 DNA to the replicative form (RF) is inhibited by rifampicin, but not by chloramphenicol (Brutlag et al.,1971). The RNA species necessary for initiation has been isolated and is designated O - RNA (Messer et al., 1975).

Tippe-Schindler et al., (1979) have therefore suggested a dual mechanism for the control of initiation of replication, comprised of one control system for the synthesis of O-RNA, and a second for the synthesis of initiation proteins (Fig. 1.7). These components then form the 'replication complex' on which DNA replication may take place. Synthesis of O-RNA itself is under two controls,

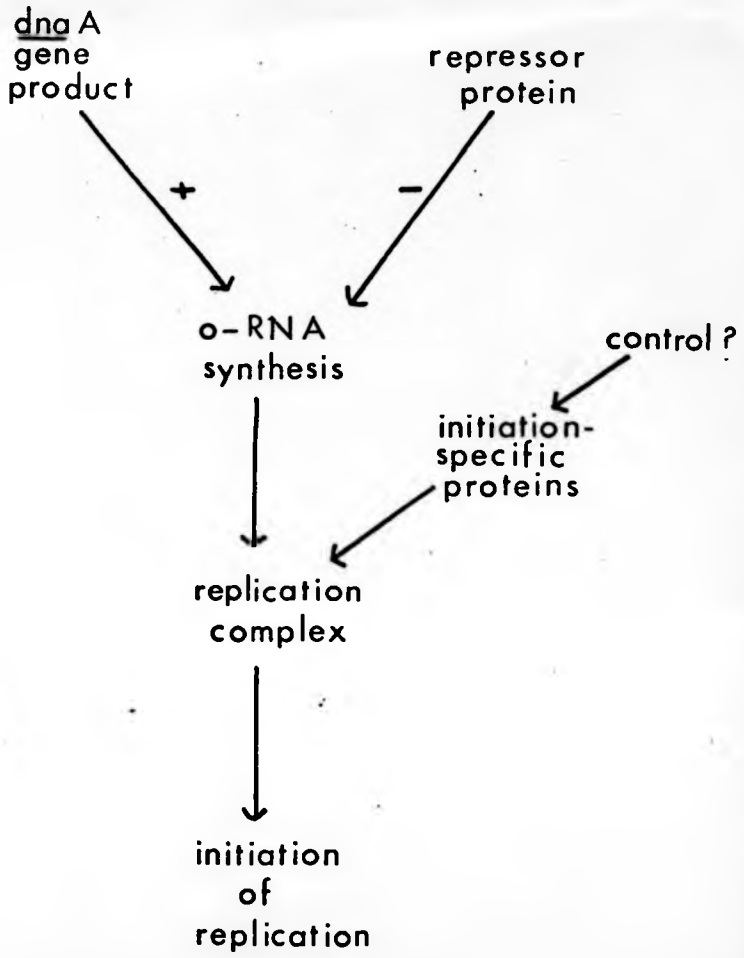


Fig. 1.7. Proposed mechanism for the control of initiation of DNA replication in E.coli*

A dual mechanism is proposed, in which both the synthesis of O-RNA and the synthesis of initiation proteins are regulated. O-RNA synthesis is controlled positively by the dnaA gene product and negatively by an unknown repressor protein.

* from Tippe-Schindler et al. (1979)

one positively-acting in which the dnaA gene product is directly involved, and the second a negatively-acting repressor protein acting after the start of transcription. The use of specific merogenotes (cells which carry an extra dnaA allele on an episome) has confirmed the role of the dnaA gene product in replication as a positively-acting control (Zahn and Messer, 1979). The merogenote over-produces O-RNA and over-initiates, indicating that the mechanism for positive control is gene dosage-dependent, and switch-off occurs under normal conditions.

1.3.5. Conclusions. As is apparent, DNA replication mutants have been invaluable in helping to elucidate the control of initiation of replication. Although there are still many unanswered questions, the information from E.coli on the control of DNA replication is applicable to many other organisms, in particular those used in studies of cellular morphogenesis and differentiation. Although E.coli is a very important experimental organism, its usefulness in studies of morphogenesis and differentiation is limited, because of its vegetative monomorphic cell cycle. It is for this reason, that attention has turned to other, more complex differentiating prokaryotes, one group of which is the sporulating Bacilli.

1.4. The endospore-forming Bacilli.

1.4.1. General morphology and the differentiation sequence in Bacillus subtilis. The endospore-forming Bacilli are a group of Gram-positive, rod-shaped bacteria, which are able to form heat-resistant endospores in unfavorable environments. Bacillus subtilis has been used for most sporulation studies since the biochemistry, and particularly the genetics of this species have been studied in depth.

The process of sporulation is manifested in a series of clearly-defined, morphological changes, which have been designated as discrete

stages in the differentiation sequence (Fig. 1.8). At the end of exponential growth, an axial filament of DNA forms parallel to the long axis of the cell. A septum forms at right angles to this in an asymmetric position, the smaller cell produced by the division being termed the prespore. Engulfment of the prespore occurs to form the forespore, spore-specific coat materials are laid down, and after a period of maturation, the spore is released.

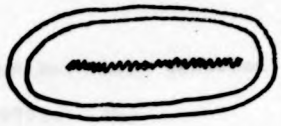
Fundamental control processes may be different in the vegetative cells of B.subtilis, from those in E.coli, for it has been shown that, unlike E.coli, cell division and DNA replication in B.subtilis are independent of each other (Donachie et al., 1971). When DNA synthesis is blocked, cell division continues, and eventually enucleate cells are produced. It is possible that in B.subtilis parallel independent pathways exist without a 'master timer' and another process may be the overriding factor in the control of cell division. RNA synthesis is required for the initiation of chromosome replication (Murakami et al., 1976), as in E.coli. The RNA is probably required as a primer for DNA polymerase, in a similar way to that in E.coli.

1.4.2. Sporulation mutants. The progress which has been made in the understanding of sporulation was possible because many sporulation mutants had been isolated and characterized. However, the inherent complexity of the system has meant that progress has been slow. The major problem is that biochemical changes which are detected on sporulation could be either sporulation-specific changes, or merely a 'shift-down' response at the end of exponential growth. In very few cases can these be distinguished.

Fig. 1.8. Stages of sporulation in B.subtilis

Spore stages	
0	End of exponential growth
I	Axial filament
IIa	Septum formation
IIb	Prespore formed
IIIa	Engulfment of prespore
IIIb	Fore-spore formed
IV	Cortex formation
V	Coat deposition
VI	Spore maturation
VII	Spore release

O



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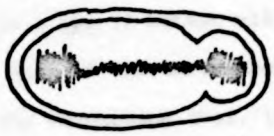

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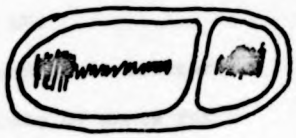

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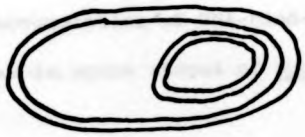
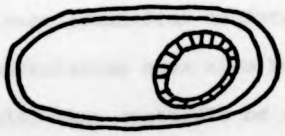


IIIb



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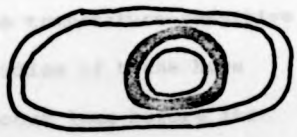


V

VI

VI

In all, several hundred loci for sporulation have been detected with the use of asporogenous mutants (Piggott, 1973). The sporulation mutants can be divided into early and late mutations, depending on the time in sporulation when they block the sequence. Each is designated by the latest stage to which it can develop, for example a spo II mutant will develop up to stage II.

Most of the early sporulation genes are found to be scattered over the chromosome (Doi, 1977b), but the clustering of genes for stages II and III has been reported (Hoch, 1974). Sporulation mutants at stage 0 are often found to have pleiotropic effects. These mutations could be mediated through an effect on membrane function, and this effect may also be reponsible for the accumulation of DNA-binding proteins seen in vegetative cells, as well as in spore stages of spo 0 mutants (Brehm et al., 1975).

In the later stages of sporulation several biochemical markers appear, such as dipicolinic acid, alkaline phosphatase, and resistance to chemicals and heat. None of the late sporulation gene functions have been identified to date. Mutants which are temperature-sensitive for sporulation have also been isolated, and studies of these have revealed that synthesis of a gene product may occur long before its physiological function is required, for example coat protein may be detected antigenically at stage II, but its function is not required until stage V (Wood, 1972). The availability of this comprehensive array of mutants has enabled an analysis of the biochemistry of sporulation to be made. A major problem in the use of spo mutants is that mutations are pleiotropic, indicating that the mutation could be in a control region rather than in a structural gene. No primary product of a spo locus has as yet been identified (Piggott and Coote, 1976).

1.4.3. Control of sporulation at the molecular level. A final round of DNA replication and cell division is necessary before forespore septum formation (Mandelstam et al., 1971). DNA polymerase activities in sporulating B.subtilis show marked differences from vegetative cells. DNA polymerase I activity increases during the early stages of sporulation, and then decreases, whereas the levels of DNA polymerases II and III do not change (Honjo et al., 1976). The increased DNA polymerase I activity indicates that the capacity for repair synthesis is increased during sporulation. DNA polymerase III activity increases significantly on spore activation, prior to the onset of replication (Ciarrochi et al., 1977).

With the use of specific inhibitors of transcription it was shown that continued transcription is necessary throughout sporulation (Leighton and Doi, 1971). Pulse labelling and inhibitor studies demonstrated that sporulation mRNA has a short half-life, and that the synthesis of enzymes associated with sporulation is also dependent on short-lived mRNA (Linnett and Tipper, 1976). Initiation of sporulation is also very sensitive to netropsin, which inhibits RNA synthesis by binding to (A-T)-rich regions of the DNA (Wartell et al., 1974). This observation led to the suggestion that early sporulation genes lie in (A-T)-rich regions of the chromosome (Keilman et al., 1975).

When sporulation begins, RNA synthesis drops by 90%, and this is followed by fluctuating levels of synthesis throughout sporulation. Simultaneously with the drop in RNA synthesis, high concentrations of ppGpp and pppGpp (Magic Spots 1 and 2) accumulate rapidly, and then decrease equally rapidly (Rhaese et al., 1975). These highly phosphorylated nucleotides (HPN) have been implicated in the control of rRNA synthesis (Van Ooyen et al., 1976). Rhaese and Groscurth (1974)

found that ppApp and pppApp (HPN I and II) are synthesized by B.subtilis ribosomes after glucose depletion or amino-acid starvation. A third HPN, pppAppp was shown to be synthesized in the membranes, and was implicated in the recognition of external conditions (Rhaese and Groscurth, 1976). p_3Ap_3 affects the translational capacity of the ribosomes so that p_2Ap_2 and p_3Ap_2 are made instead of p_2Gp_2 and p_3Gp_2. p_3Ap_3 synthetase resides in the membrane, is inhibited by energy sources such as glucose, and is coded for by the spoOF gene (Rhaese and Groscurth, 1979). Temperature-sensitive mutations in this gene cause the abolition of sporulation, and they also have a temperature-sensitive p_3Ap_3 synthetase, suggesting that p_3Ap_3 is a sensor molecule for the initiation of sporulation. Significant levels of cAMP have not been observed in most Bacilli tested (Setlow, 1973), but cGMP has been found (Bernlohr et al., 1974).

Competition DNA-RNA hybridization experiments showed that sporulation-specific RNA's are transcribed from both heavy and light strands in a sequential manner during sporulation (Dicioccio and Strauss, 1973). However, even at the late stages of sporulation, 60% of mRNA is qualitatively vegetative phase mRNA (Linn and Losick, 1976). Inherent problems in these mRNA studies are the inability to study a gene-specific mRNA, and the lack of quantitation of mRNA species.

There is an increasing body of evidence to suggest some degree of control over sporulation exists at the level of translation, but far less attention has been paid to it compared with studies of transcriptional control. The stability of mRNA during sporulation has important implications for the extent of translational control but unfortunately the evidence available on this point is conflicting. Some studies suggest there are stable mRNA molecules (Sterlini and Mandelstam, 1969),

whereas others find them to be unstable (Leighton, 1974). Most evidence is obtained through the use of RNA synthesis inhibitors, the main difference between individual studies being the concentrations used. The use of low concentrations suggests that mRNA is stable, but these concentrations may allow residual RNA synthesis to occur, whereas high inhibitor concentrations suggest that mRNA is unstable, but there is evidence that the inhibitor rifampicin, which is most commonly used, causes some cell lysis even at low concentration (1 $\mu g ml^{-1}$) (Coote et al., 1973). The use of mutants tends to suggest that mRNA is short-lived (Leighton and Doi, 1971).

The demonstration of a change in the specificity of translation is necessary to establish the existence of translational control. In vitro translation experiments showed that 'idling' vegetative ribosomes produce the highly phosphorylated nucleotides p_2Gp_2 and p_3Gp_2, whereas ribosomes from sporulating bacteria produce p_2Ap_2 and p_3Ap_2 (Rhaese and Groscurth, 1974). This difference may reflect a functional change in translation during sporulation. An active in vitro protein synthesizing system from B.subtilis was able to translate adequately bacteriophage SPO-1 RNA in the presence of initiation factor (IF) from vegetative cells, but in the presence of IF from sporulating cells this ability was greatly reduced. This effect was a definite change in template specificity rather than a general loss of activity (Chambliss and Legault-Demare, 1975).

Post-translational control of sporulation has been demonstrated in the formation of the spore coat protein. Coat protein is deposited at stage V, but Wood (1972) was able to detect the protein immunologically as early as stage II. The protein could also be detected in spo mutants blocked at stage II. This evidence suggests a specific mechanism is

operating to control the assembly of accumulated coat proteins into the final spore coat. There is some evidence that post-translational modification may also occur by limited proteolysis (Sad off et al., 1970), but the significance of this is unclear.

In summary, it has been shown that differential gene expression occurs during sporulation, and control mechanisms are operating primarily at the level of transcription, although some translational control can be demonstrated.

1.4.4. RNA polymerase and its involvement in transcriptional control. Since transcriptional control plays an important role in the regulation of sporulation, the RNA polymerase enzyme present during sporulation has been extensively investigated. The vegetative RNA polymerase holoenzyme is made up of 5 polypeptides, α_2, B, B' and σ, to which the smaller polypeptide ω is often attached (Berg and Chamberlin, 1970). The core enzyme of α_2BB' is functional in RNA chain elongation, but fails to initiate from a natural initiation site.

The initial observation, that RNA polymerase loses its ability to transcribe bacteriophage Øe DNA early in sporulation, led to the proposal that a change in template specificity of the enzyme occurs at the onset of sporulation (Brevet and Sonenshein, 1972). This issue has remained controversial, since the change could be a characteristic of stationary phase RNA polymerase rather than a reflection of sporulation-specific changes (Szulmajster, 1973). The study of functional RNA polymerase mutants has complemented biochemical analysis of the enzymes' subunit structure.

In a converse approach to the isolation of sporulation mutants, specific functional RNA polymerase mutants have been selected. The most useful of these are mutants temperature-sensitive at various stages of sporulation, which are able to grow and sporulate at the

permissive temperature, and grow but fail to sporulate at the restrictive temperature (Leighton, 1973). The RNA synthesis inhibitor rifampicin has been shown to inhibit transcription by binding to the β-subunit of RNA polymerase (Rabussay and Zillig, 1969). The effect of rifampicin on RNA polymerase activity in the mutant vegetative cells was different from that on the enzyme activity in mutant sporulating cells, suggesting that the enzyme was modified during sporulation (Sumida-Yasmumoto and Doi, 1977).

The hypothesis (Losick et al., 1970), that modification of the β-subunit occurred on the transition to sporulation, was proved incorrect when it was shown that the modification was the result of proteolysis during the purification procedure (Linn et al., 1973). The problem of proteolysis has plagued subsequent biochemical investigations on the enzyme, resulting in considerable controversy. Early experiments reporting alterations in the σ-factor were in fact done under conditions where proteolysis could occur (Sonenshein and Losick, 1970), and later data shows that σ-factor from sporulating and vegetative cells, and also asporogenous mutants, antigenically cross-react (Duie et al., 1974).

Recently it was found that the core structure of RNA polymerase may be modified by association of the core with polypeptides other than the σ-factor (Fukuda and Doi, 1977). During forespore formation two forms of RNA polymerase were identified. One (enzyme I) is identical to the holoenzyme from vegetative cells, but the second (enzyme II) has a subunit designated δ instead of σ. The new subunit has a molecular weight of 28,000 daltons. Enzyme I is much less sensitive to netropsin than enzyme II, suggesting that the enzymes have different binding sites on the DNA.

Regulatory mechanisms other than RNA polymerase modification may be involved in the control of transcription. Transcription of the *lac* operon requires cyclic AMP, cyclic AMP receptor protein and a repressor protein in addition to RNA polymerase, for adequate transcription (De Crombrugghe *et al.*, 1971). Regulatory attenuator sites which regulate the termination of transcription in a region of the operon preceeding the structural gene have been identified in the *trp* gene (Bertrand *et al.*, 1975).

1.4.5. *Germination of endospores*. In comparison with sporulation, experimental studies on the germination of endospores has been neglected. Although much is known about the external factors which influence spore germination, little work has been done on the biochemistry of the process. The difference between spore germination and the vegetative state is a reflection of the limited classes of protein synthesized during germination. Gene position with respect to the chromosomal origin does not dictate the time of enzyme synthesis, i.e. sequential gene transcription does not occur (Yeh and Steinberg, 1977).

Contradictory reports concerning the nature of RNA synthesized during germination have appeared in the literature. Early workers found that only rRNA is synthesized early in germination (Armstrong and Sueoka, 1968), but their analysis was based on sucrose gradients, which do not adequately distinguish mRNA and rRNA populations. Recent data shows that pulse labelled RNA from vegetative and germinating cells appear quite similar on polyacrylamide gels (Sloma and Smith, 1979), suggesting that there are no transcriptional controls which are specific to spore germination. Certain individual RNA transcripts however, are unique to the germination process (Margulies *et al.*, 1978).

1.4.6. Genetic analysis of B.subtilis. A major reason for the use of B.subtilis as a model for differentiation is the extensive genetical background already available. Not only is the range of sporulation mutants very extensive, but both transformation and transduction have been demonstrated in the bacterium, making it amenable to genetic manipulation. Genetic analysis has permitted the construction of a B.subtilis genetic map, which although not as detailed as that of E.coli, has proved to be very useful.

B.subtilis cells become competent for transformation towards the end of the exponential growth phase, the competent cells being physiologically different from non-competent cells. For example,they become resistant to penicillin for some time after transformation (Nester, 1964). B.subtilis can take up double-stranded DNA, and also single stranded DNA in the presence of EDTA (Chilten and Hall, 1968), and after transformation donor DNA can be retrieved in a covalent association with recipient DNA (Bodmer and Ganesan, 1964). Davidoff-Abelson and Dubnau (1971) have suggested that the systems utilized in repair and recombination and in transformation may be similar, since in rec A mutants, the transformation frequency was 10-25% of that in wild type cells.

Plasmids have been isolated and characterized from B.subtilis and B.cereus, with a view to the plasmids' use as potential gene cloning vectors (Tanaka et al., 1977; Bernhard et al., 1978). In B.cereus, bacteriocin production and tetracycline resistance can be attributed to two plasmids, but the B.subtilis plasmids do not show any detectable phenotypic traits.

Temperate phage for B.subtilis have been investigated, also in view of their potential as cloning vectors. The best characterised is

ø105, a lysogenic phage for which there is a physical genetic map (Birdsell et al., 1969). A closely related phage ρ14 (Dean et al., 1978) was found to have only one site for the restriction endonuclease Bgl II, and therefore has more potential as a cloning vector than ø105, which has multiple sites for most restriction enzymes. ρ14 is presently being developed as a cloning vector.

It appears that the range of natural cloning vectors in B.subtilis is limited, but it is possible to transfer plasmids from Staphylococcus aureus to B.subtilis (Erlich, 1977). These small plasmids are more suitable cloning vectors than the native Bacillus plasmids. A hybrid plasmid between pC194 (a Staphylococcus plasmid) and the E.coli plasmid pBR322 may be used as a cloning vector in both hosts (Erlich et al., 1978).

1.4.7. Conclusions. Sporulation in B.subtilis is a good system in which to study the control of morphogenesis and differentiation. Its major advantages are the multitude of asporogenous mutants which have been isolated and characterised, and the availability of a genetic map. Investigation of the molecular biology of sporulation has been controversial, and has lagged behind genetic studies, primarily because of the most important disadvantage of the system, namely the inability to distinguish between biochemical changes due to shift-down at the end of exponential growth, and changes which are truly sporulation-specific. This problem has hampered research for a long time, and a solution to it has not yet been found. Another disadvantage is the pleiotropic nature of most sporulation mutants. The application of DNA cloning techniques to studies of sporulation may allow the expression of individual sporulation genes to be examined in isolation (Segall and Losick, 1977), and then further progress in this fascinating area may be made.

Other, less well-known bacteria have been recently used in studies of morphogenesis and differentiation, and one of these is the prosthecate bacterium Caulobacter crescentus.

1.5. The prosthecate bacterium Caulobacter crescentus

1.5.1. The Caulobacter cell cycle.

C.crescentus is a prosthecate bacterium, that is one which possesses a cellular extension, the membrane of which is continuous with the cytoplasmic membrane of the cell. The prostheca, or stalk, is an integral part of the cell.

An exponential culture contains three cell types. These are a stalked or 'mother' cell which has a polar prostheca, a swarm or 'daughter' cell, which has a polar flagellum and pili, and a pre-divisional cell with the characteristics of both the swarm cell and the stalked cell. The swarm cell differentiates into a stalked cell, and then in the pre-divisional cell, swarm cell characteristics appear at one pole (Fig. 1.9). Division of the pre-divisional cell is asymmetric, leading to two distinct cell types. The stalked cell, immediately after division, can initiate a new round of growth and division to produce another swarm cell. The swarm cell however, must undergo a period of differentiation to become a stalked cell before it can reproduce (Shapiro, 1976).

The distinctive sequential, morphological changes which mark this differentiation sequence make this prokaryote an attractive alternative for studies of cellular morphogenesis and differentiation. A second important advantage of this organism is that populations of synchronous cells can be obtained by differential centrifugation (Stove and Stanier, 1962), or by adhesion of the cells via a holdfast to petri dishes or membranes (Swoboda and Dow, 1979). In this latter method the membrane

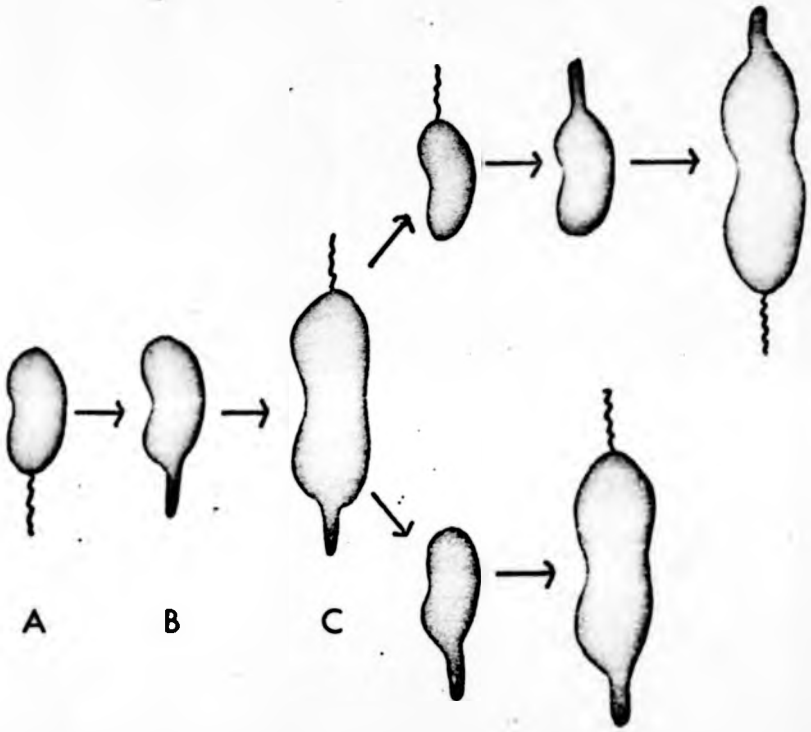


Fig. 1.9. The dimorphic cell cycle of C.crescentus*

A: the motile swarm cell bearing a single flagellum

B: the non-motile stalked cell

C: the dividing cell, which possesses a stalk at one pole and a flagellum at the other.

Asymmetric cell division gives rise to a swarm cell and a stalked cell, the stalked cell proceeding directly to growth and cell division, whereas the swarm cell must undergo a period of maturation and differentiation to a stalked cell, prior to further reproduction.

* From Degnen and Newton (1972b)

is washed repeatedly with growth medium, and after several minutes the only free cells are found to be swarm cells released after division of the attached stalked cells (Fig. 1.10). These are harvested and are usually at a concentration of 10^7 ml^{-1} (Table 1.2). For any biochemical studies of the cell cycle a population of synchronous cells is essential, since this allows amplification of the biochemical changes occurring in each individual cell, and thus facilitates their study.

1.5.2. The kinetics and regulation of macromolecular synthesis during the cell cycle. The use of synchronous cultures enabled detailed biochemical studies to be undertaken on the C.crescentus differentiation cycle. DNA replication is a characteristic of the stalked cell only, with a G_1 period (or maturation period) of approximately 65 min. in the swarm cell. Replication in the stalked cell shows no such presynthetic gap (Degnen and Newton, 1972b; Fig. 1.11), but recent evidence indicates that there may be a small G_1 in the stalked cell cycle (Swoboda et al., in press). Shapiro (1976) suggested that there are two programmes for gene expression in C.crescentus; one in the stalked cell, and one in the swarm cell to stalked cell transition. It is possible that these may be mediated by modified RNA polymerases as may be the case in B.subtilis, but no differences in RNA polymerases from each cell type have been found (Bendis and Shapiro, 1973).

The use of specific DNA synthesis inhibitors such as mitomycin c, enabled Degnen and Newton (1972a) to show that cell division was dependent upon the completion of DNA replication as for E.coli (Clark, 1968). It appears that a dependent pathway of cell cycle events operates in C.crescentus, in which DNA replication, cell division and stalk formation are directly linked (Terrana and Newton, 1976), with

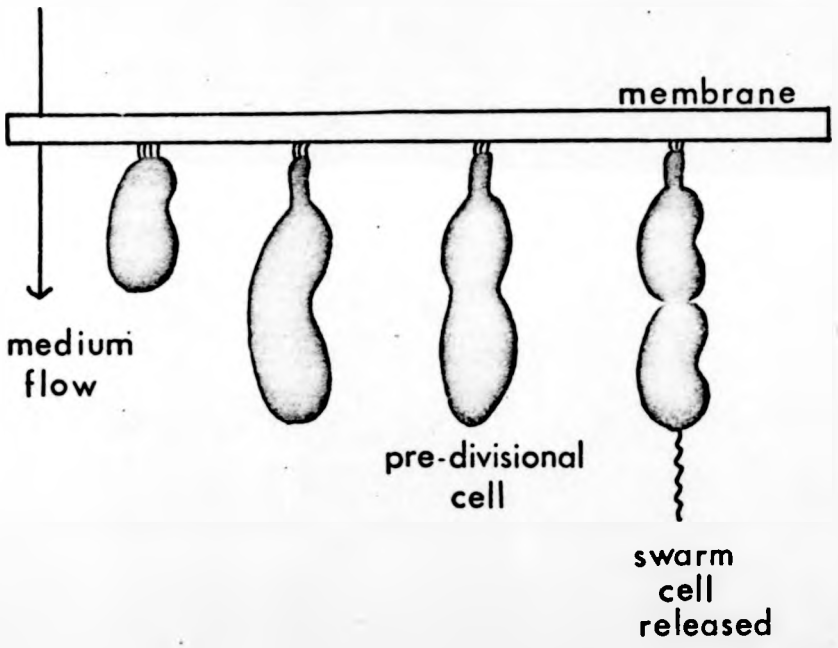


Fig. 1.10. Successive stages in the life cycle of an individual C.crescentus cell attached to a membrane filter*

Homogeneous swarm cell populations are selected by filtration, and then are allowed to attach by the holdfast to an inverted membrane filter. They differentiate to become stalked mother cells and release newly formed swarm cells into the medium at regular intervals.

* From Swoboda and Dow (1979)

Table 1.2. Cell yields by selective synchronization techniques

Organism	Synchronization procedure	Volume of synchronized culture	Viable cells/ml	Reference
Caulobacter	i) repeated centrifugation	2-3 ml	not given	Stove and Stanier (1962)
"	ii) adhesion of mother cell	25 ml	$1\text{-}3 \times 10^7$	Degnen and Newton (1972b)
Hyphomicrobium	centrifugation and filtration	30 ml	2×10^7	Moore and Hirsch (1973)
E.coli	density gradient centrifugation	1-2 ml from a 15 ml gradient	4×10^8	Mitchison and Vincent (1965)
Bacillus subtilis	filtration	-	1-2% of initial population	Sargent (1973)
Rhodopseudomonas palustris	density gradient centrifugation	2-3 ml	1.4×10^9	Westmacott and Primrose (1976)
Rhodomicrobium vannielii	filtration	100 ml - 15 litres	$1\text{-}2.5 \times 10^7$	Whittenbury and Dow (1977)

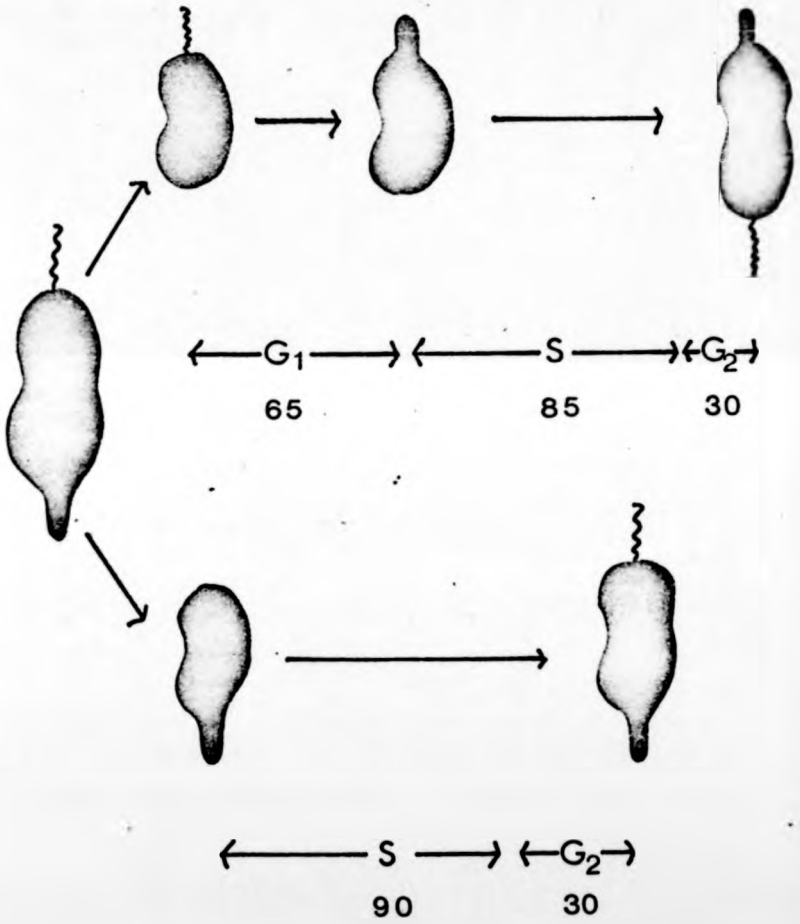


Fig. 1.11. The DNA cycles in swarmer and stalked cells of C.crescentus*

Numbers are the average time spent in each phase (min).

* From Degnen and Newton (1972b)

RNA synthesis a necessary prerequisite for the initiation of DNA replication (Newton, 1972). A non-motile, temperature-sensitive mutant was used to establish that neither flagellin synthesis nor assembly are required for cell division(Olsey and Newton, 1977). Shapiro (1976) postulated that flagellin synthesis and flagellar assembly form a secondary, ancillary pathway independent of the progress of the cell cycle.

In C.crescentus, 1% of RNA is polyadenylated with poly A tracts of 15-50 nucleotides, but these RNAs are unstable,and are made at all stages of differentiation (Ohta et al., 1978). However, they may have a role in post-transcriptional control as in eukaryotes (Darnell et al., 1974).

Protein synthesis during C.crescentus differentiation was examined by pulse labelling synchronous cultures with ^{35}S-methionine, followed by analysis using one and two-dimensional polyacrylamide gel electrophoresis. Proteins specific to the swarm cell and to the stalked cell were identified, indicating that differential gene expression operates during differentiation (Cheung and Newton, 1977). Measurements of the absolute rates of protein synthesis and of the half-lives of mRNA populations showed that the changes in protein patterns were the result of transcriptional control (Iba et al., 1978). This work has recently been extended to an examination of the products of cell-free, transcription-translation systems using isolated C.crescentus nucleoids (Evinger and Agabian, 1977) from different developmental stages. The proteins synthesized from mRNA attached to the nucleoid at the time of preparation were examined, and stage-specific membrane proteins were identified (Evinger and Agabian, 1979).

Translational studies in C.crescentus have focussed on the initiation of protein synthesis, using isolated ribosomes and the natural mRNAs provided by RNA phage. C.crescentus ribosomes are unable to bind and translate mRNA from the coliphage MS2, whereas translation of homologous mRNA from the C.crescentus phage ØCb5 is efficient (Leffler and Szer, 1973). This specificity resides in the 30S subunit. Several initiation factors have been isolated from C.crescentus, and all have been found to be interchangeable with E.coli initiation factors, and so these are not species-specific (Leffler and Szer, 1974).

1.5.3. Genetic analysis of C.crescentus. C.crescentus has proved to be amenable to genetic analysis, increasing its usefulness in morphogenesis and differentiation studies. Auxotrophic mutants can be obtained and used to demonstrate conjugation and the transfer of genetic markers between strains (Newton and Allebach, 1975). Temperature-sensitive cell cycle mutants have proved useful in the study of cell cycle control (Kurn et al., 1974), as described earlier in the elucidation of the relationship between flagellin synthesis and cell division (Olsey and Newton, 1977).

Two transducing phage for C.crescentus have been isolated (Ely and Johnson, 1977), but unusually, both are virulent and do not lysogenize the host. Drug resistance plasmids such as the inc-P plasmid RP4 have been transferred to C.crescentus to allow genetic analysis (Ely, 1979). The plasmids are stably maintained and can be used to promote chromosomal exchange between C.crescentus strains. Use of such plasmids may also allow the introduction of transposons into the chromosome for further genetic analysis.

1.5.4. Conclusions. C.crescentus is a very useful model for studying morphogenesis and differentiation since it shows an obligate, well-defined series of morphological changes, synchronous populations of swarm cells may be obtained, and it is amenable to genetic analysis. However, it does have limitations. The first is that the normal sequence of differentiation is not easily manipulated by external factors, which means that controlling the 'switch on-off' of key mechanisms is difficult. Nonetheless, the C.crescentus cell does have the ability to assess the nutritional status of the environment, since a lack of carbon arrests the cells at the pre-divisional stage. Cyclic AMP has been implicated in this mechanism (Kurn et al., 1977), but highly phosphorylated nucleotides have not been found. A second major disadvantage is that only small volumes of swarm cells may be synchronized at one time (25 ml, 10^7 ml^{-1}; Table 1.2), so that large-scale biochemical analyses are difficult.

1.6. The Rhodospirillaceae (purple non-sulphur bacteria)

1.6.1. The budding bacteria. Several members of the Rhodospirillaceae may be classified as 'budding bacteria', and show obvious potential for studies of morphogenesis and differentiation. Cell separation in the budding bacteria results in two asymmetric cells, whereas in E.coli, division results in two symmetrical siblings. In both cases however, division is by binary fission.

The budding bacteria show polar growth, irrespective of the nutrient conditions, and this can be uni- or bi-directional, and from either pole (Fig. 1.4). A consequence of polar growth for example in R.vannielii, is that the daughter cell will be composed primarily of new cellular material. This introduces the concept of aging, since little new material is added to the mother cell with

successive rounds of division. The process of obligate polar growth also confers the capacity for morphogenetic evolution, which is not possible in bacteria which grow by intercalary processes, and increasing degrees of morphogenetic complexity can be traced through the budding bacteria (Fig. 1.12). The consequent polarization of the cell may have resulted in the need for regulation of temporally related morphogenetic events, making the budding bacteria an ideal group in which to study morphogenesis and differentiation.

1.6.2. The Rhodospirillaceae as models for cellular morphogenesis and differentiation. The purple non-sulphur bacteria are a group of Gram-negative, photosynthetic bacteria which reproduce by a 'budding' process. The particular interest in the Rhodospirillaceae lies not only in their use in studies of morphogenesis and differentiation, but also results from their ability to fix CO_2 and N_2. They may be grown photoheterotrophically under anaerobic, light conditions, and chemoheterotrophically in the dark, under aerobic conditions. Members of the group have been used in studies of bacterial photosynthesis and also nitrogen fixation (e.g. Rhodospirillum rubrum, Schick, (1971)).

Since the Rhodospirillaceae obtain energy from light, the energy status of cultures can be easily regulated during experiments. Photopigments are synthesized de novo in the daughter cells, in response to light, and so the organisms are far more amenable to physiological manipulation than for example, C.crescentus or Hyphomicrobium, another budding bacterium.

Two members of the Rhodospirillaceae, Rhodopseudomonas palustris and Rhodomicrobium vannielii have been exploited for studies of cellular morphogenesis and differentiation. Since R.vannielii is the subject of this thesis, it is considered in detail in 1.7.

Fig. 1.12. Budding bacteria ordered in degrees of morphological/cell cycle complexity*

a. Rhodopseudomonas acidophila

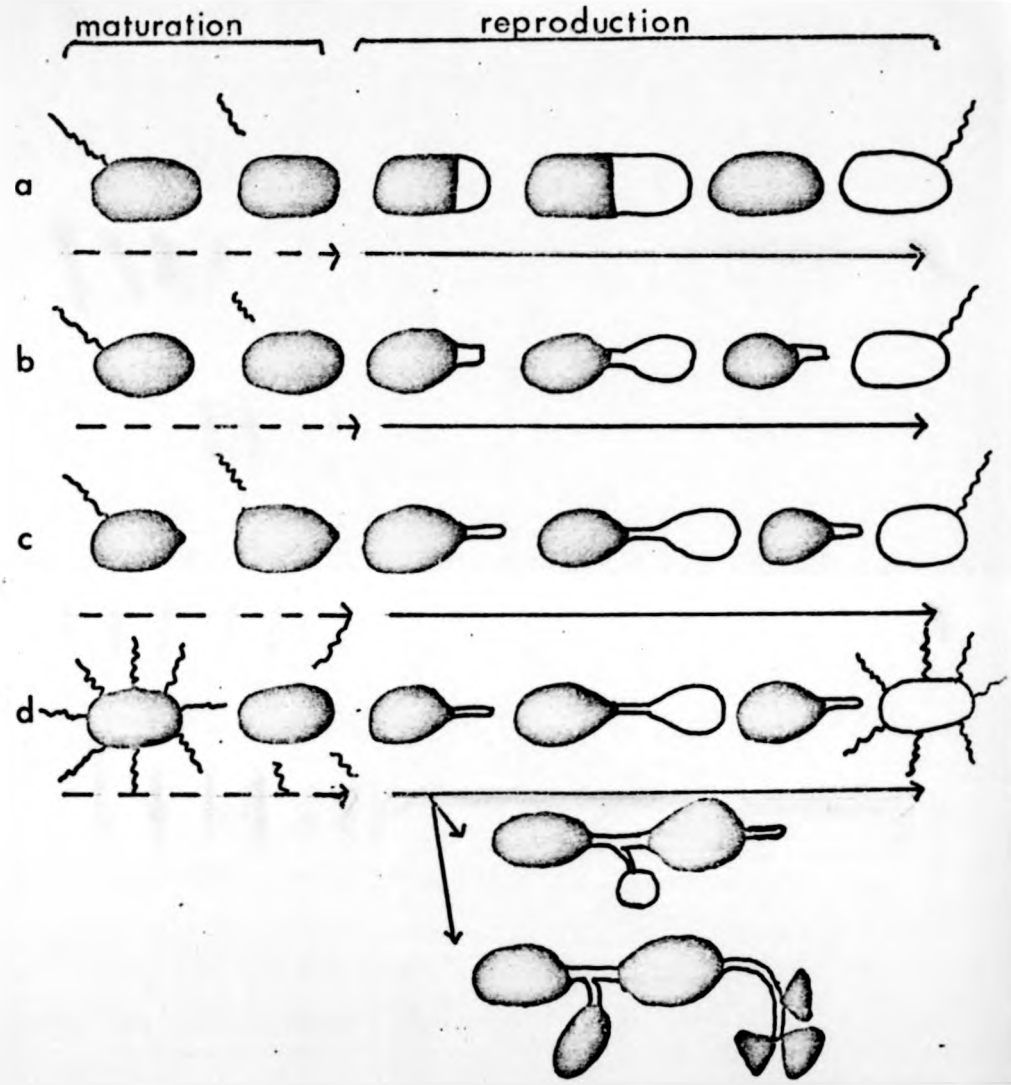
b. R.palustris/viridis

c. Hyphomicrobium sps

d. Rhodomicrobium vannielii

In all cases daughter cell synthesis is by obligate polar growth, and asymmetric cell division gives rise to an immature daughter cell and a mature mother cell.

* From Whittenbury and Dow (1977)


maturation
reproduction
a
b
c
d

Experiments on the cell cycle of R.palustris showed that DNA replication is a characteristic of the stalked cell only (Westmacott and Primrose, 1976; Fig. 1.13). Cell division, flagellum and holdfast synthesis are dependent upon the completion of chromosome replication (Westmacott and Primrose, 1977), indicating a 'dependent pathway' method for the regulation of cell cycle events.

R.palustris has two major drawbacks as a model system for morphogenesis and differentiation. The first is that synchronous populations of swarm cells are selected by sucrose gradient centrifugation, and consequently large quantities of cells are difficult to obtain (Table 1.2; Westmacott and Primrose, 1976). The second is that the cell cycle has only two morphological 'landmark' events - the loss of a flagellum, and bud development, making the correlation of biochemical events and morphological markers difficult.

1.6.3. Genetics of the Rhodospirillaceae. The genetics of the Rhodospirillaceae, except for Rhodopseudomonas capsulata and Rhodopseudomonas sphaeroides, has been neglected. A recent review by Saunders (1978) considers the use of mutants in photosynthesis research, and the methods of gene transfer in the Rhodospirillaceae. The importance of work on the genetics of these bacteria cannot be overestimated, if the genes responsible for photosynthesis and nitrogen fixation are to be identified. Similarly the processes controlling morphogenesis and differentiation can be examined by genetic techniques. Mutants of several photosynthetic bacteria have helped elucidate a number of metabolic pathways, notably the electron transport pathways (Picorel et al., 1977), and bacteriochlorophyll synthesis (Lascelles, 1975).

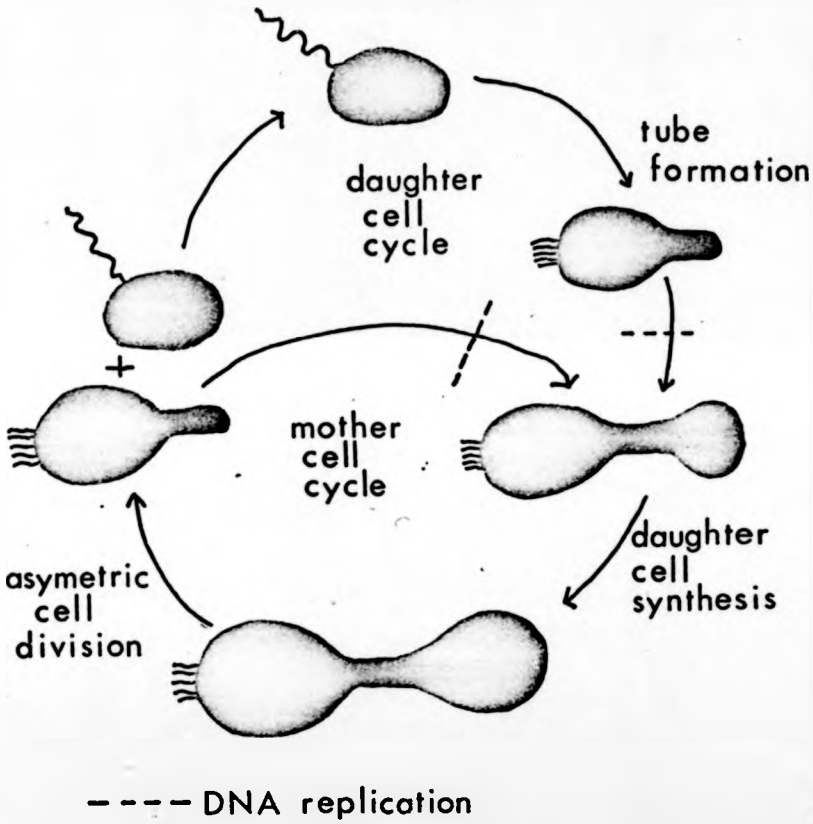


Fig. 1.13. DNA replication in the cell cycle of Rhodopseudomonas palustris*

The motile 'swarm' cell sheds its flagellum and forms a tube, and the daughter cell is synthesized at the end of the tube. Asymmetric cell division leads to the formation of a swarm cell and a 'mother' cell. The mother cell may immediately initiate a round of cell division, whereas the swarm cell first undergoes a period of maturation. DNA replication is a characteristic of the 'mother' or stalked cell only.

* From Westmacott and Primrose (1977)

1.6.3.1. Plasmids and conjugation. Plasmid DNA has been detected in several photosynthetic bacteria, but no gene functions have been assigned to the individual plasmids (Suyama and Gibson, 1966; Saunders et al., 1976), and they cannot promote conjugation. In contrast, foreign plasmids, notably those of the 'P' incompatibility group, have been shown to promote gene transfer between photosynthetic bacteria. Miller and Kaplan (1978) demonstrated the transfer of RP4 from E.coli to R.sphaeroides, and showed that the plasmid could be stably maintained. R.68.45 (a derivative of RP4 which mobilizes chromosomal fragments more efficiently) has been transferred to, and maintained in R. sphaeroides (Sistrom, 1977). In addition Tucker and Pemberton (1979a) have been able to transfer an RP4-Mu plasmid to R.sphaeroides, the highly efficient insertion system of Mu being used to increase the frequency of transfer by 100-fold. The importance of introducing foreign plasmids into the cell is that they are then able to mobilize pieces of the host chromosome, allowing genetic analysis of the unique characteristics of the organisms.

1.6.3.2. Transformation. There has been no unequivocal demonstration of transformation in the photosynthetic bacteria, although it has been shown in the cyanobacteria (Herdman and Carr, 1971). Transformation could be unsuccessful because of the production of extracellular nucleases by the recipient bacterium, but this was shown not to be the case for at least one R.sphaeroides strain (Saunders, 1978). Another explanation for the lack of transformation could be that the cell wall is not competent to take up DNA. Studies with R plasmid-directed gene transfer and the 'Gene Transfer Agent' (1.6.3.3.) indicate that the Rhodospirillaceae are recombination-proficient.

1.6.3.3. Transduction and the 'Gene Transfer Agent'. Phage for the purple non-sulphur bacteria have been described, but bona fide transduction has not been demonstrated. The phage are virulent but not lysogenic (Mural and Friedman, 1974; Wall et al., 1975a).

Marrs (1974) described an unusual system of genetic exchange in R.capsulata. This Gene Transfer Agent (GTA) is resistant to RNase and DNase, and the gene transfer is limited to R.capsulata. The GTA was identified as a small icosahedral virus containing DNA of molecular weight 3.6×10^6 daltons (Marrs, 1977). The gene transfer process resembles that of generalized transduction, although the GTA is much smaller than any known transducing phage. The GTA has also been used to transfer nitrogenase (nif) and hydrogenase genes from the wild type to a nif^- mutant of R.capsulata (Wall et al., 1975b). Unfortunately only small fragments of DNA may be transferred because of the small size of the GTA, and so only fine genetic mapping is possible.

1.6.3.4. Conclusions. Although the use of R.palustris as a model differentiation system has been limited, the Rhodospirillaceae as a whole should prove to be very useful in the future. The mechanisms for genetic exchange in the Rhodospirillaceae are now being thoroughly explored, and will be of immense value in the elucidation of the unusual properties of this group of bacteria. The utilization of foreign plasmids in conjugation appears to offer the most promise for future research, bearing in mind that the GTA is restricted to R.capsulata.

A member of the Rhodospirillaceae which has not been discussed above is R.vannielii. The characteristics of this bacterium are described in detail in 1.7., but it should be emphasized that R.vannielii shows all the advantages of a budding bacterium in terms of morphogenesis and differentiation research.

1.7. Rhodomicrobium vannielii as a model system for cellular morphogenesis and differentiation

1.7.1. Isolation, morphology and the cell cycle. R.vannielii is the most advanced of the Rhodospirillaceae, in terms of morphogenetic complexity. It was originally isolated by Duchow and Douglas (1949), and the strain used in this work is R.vannielii strain RM5, isolated from freshwater by C.S. Dow.

R. vannielii has a polymorphic cell cycle, in which four cell types may be expressed, depending upon the environmental conditions (Fig. 1.14). In exponential batch culture two cell types are found. The first is motile swarm cells, and the second, 'mother' or stalked cells which are linked by cellular filaments, to form microcolonies. The swarm cell undergoes a period of obligate differentiation to become a stalked cell, and further reproduction produces a new microcolony. In stationary phase cultures, a third cell type, the angular exospore, is expressed. Under conditions of low light and high CO_2 concentration, the microcolony cell expression is repressed, and the cell types present in exponential culture are swarm cells, stalked cells, and pre-divisional cells. This type of vegetative cell cycle expression has been termed the 'simplified cell cycle' (Dow and France, 1980).

1.7.2. Recent research on R.vannielii. The use of R.vannielii as a model system for cellular morphogenesis and differentiation has been documented by Whittenbury and Dow (1977). Recent studies (Taylor and Dow, 1980) have investigated the photosynthetic capacity of R.vannielii in continuous culture, and elucidated the nature of ribulose bisphosphate carboxylase, the primary enzyme in CO_2 fixation.

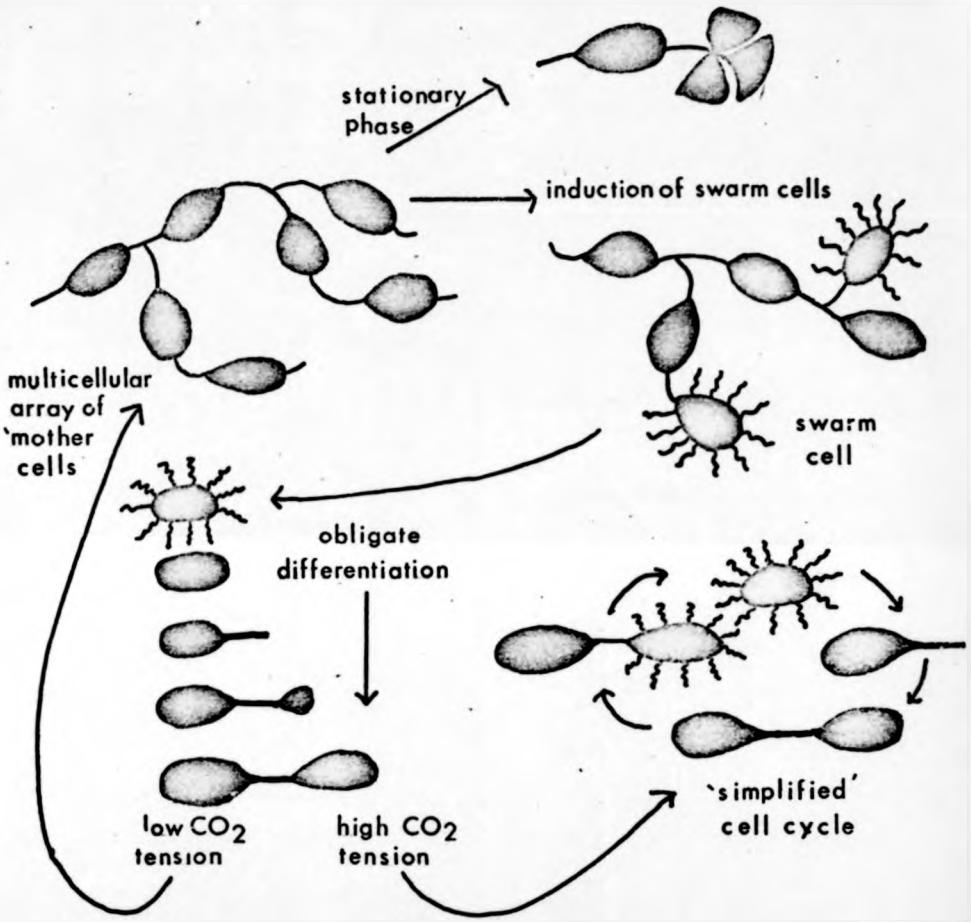


Fig. 1.14. Cellular expressions shown by R.vannielii in response to environmental stimuli

In exponential batch culture two cell types are present - 'mother' cells joined by filaments in a multicellular array or microcolony, and motile swarm cells. In conditions of high light and nutrient status, the swarm cell undergoes an obligate differentiation to form two cells. Under low CO_2 tension a new multicellular array is then developed. Under high CO_2 tension this cell cycle type is not expressed, and the 'simplified cell cycle' occurs. In stationary phase, resistant, angular exospores are formed.

An investigation of the growth characteristics and protein synthesis in the 'simplified cell cycle' expression was undertaken (France, 1978) with a view to using this simpler system to examine morphogenesis and differentiation.

Recent work has been concerned with the control mechanisms involved in morphogenesis and differentiation. A particular part of the cell cycle, the obligate differentiation of the swarm cell to the stalked reproductive 'mother' cell (Figs. 1.15 and 1.16), was chosen for more intensive study. The reasons for this were three-fold. The swarm cell undergoes a series of well-defined obligate morphological changes ('landmark' events) in the differentiation sequence (Fig. 1.17). Homogeneous populations of swarm cells can be selected by filtration quickly and in large quantities (Fig. 1.18, Table 1.2; Whittenbury and Dow, 1977). Thirdly, swarm cell differentiation is dependent on the presence of light, and so the onset of the cellular morphogenesis and differentiation sequence is easily controlled. It was established from kinetic studies of chlorophyll, protein and RNA synthesis that the swarm cell is an active metabolic unit (Whittenbury and Dow, 1977). Moreover, analysis of proteins from the swarm cell and the stalked cell showed proteins which were specific to each cell type (Fig. 1.19; Dow and France, unpublished data).

Since transcriptional control is likely to be involved in cellular morphogenesis and differentiation in R.vannielii, the role of RNA polymerase during swarm cell differentiation has been investigated. Growth of the swarm cell is found to be less sensitive to rifampicin than growth of the mother cell (Fig. 1.20.; Whittenbury and Dow, 1977). This suggests that modification of the RNA polymerase may be part of the mechanism for differential gene expression during swarm

Fig. 1.15. Light micrographs showing the obligate morphogenesis and differentiation sequence of the R.vannielii swarm cell.

Homogeneous populations of swarm cells were selected by filtration (2.12), and incubated phototrophically at 30°C. At hourly intervals, aliquots were removed and examined in the light microscope. Figures represent hours after swarm cell selection.

Magnification = 4,000 ×.

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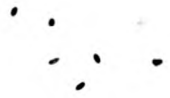

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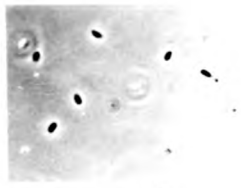

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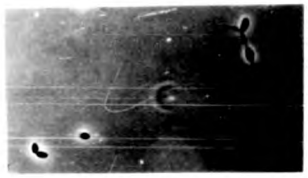

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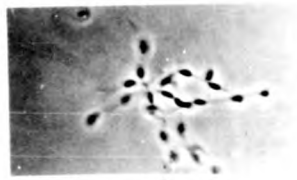

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Fig. 1.16. Electron micrographs showing the obligate morphogenesis and differentiation sequence of the R.vannielii swarm cell

Homogeneous populations of swarm cells were selected by filtration (2.12), and incubated phototrophically at 30°C. At hourly intervals, aliquots were removed, shadowed with gold-palladium wire, and examined in the electron microscope.

Figures represent hours after swarm cell selection.

Magnification = 15,000 x.

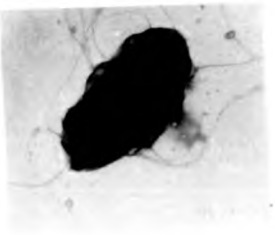
Fig. 1.16. Electron micrographs showing the obligate morphogenesis and differentiation sequence of the R.vannielii swarm cell

Homogeneous populations of swarm cells were selected by filtration (2.12), and incubated phototrophically at 30°C. At hourly intervals, aliquots were removed, shadowed with gold-palladium wire, and examined in the electron microscope.

Figures represent hours after swarm cell selection.

Magnification = 15,000 x.

hrs



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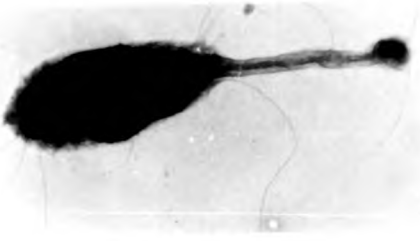

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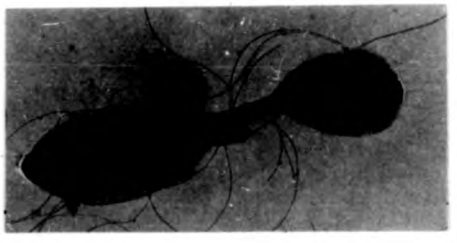

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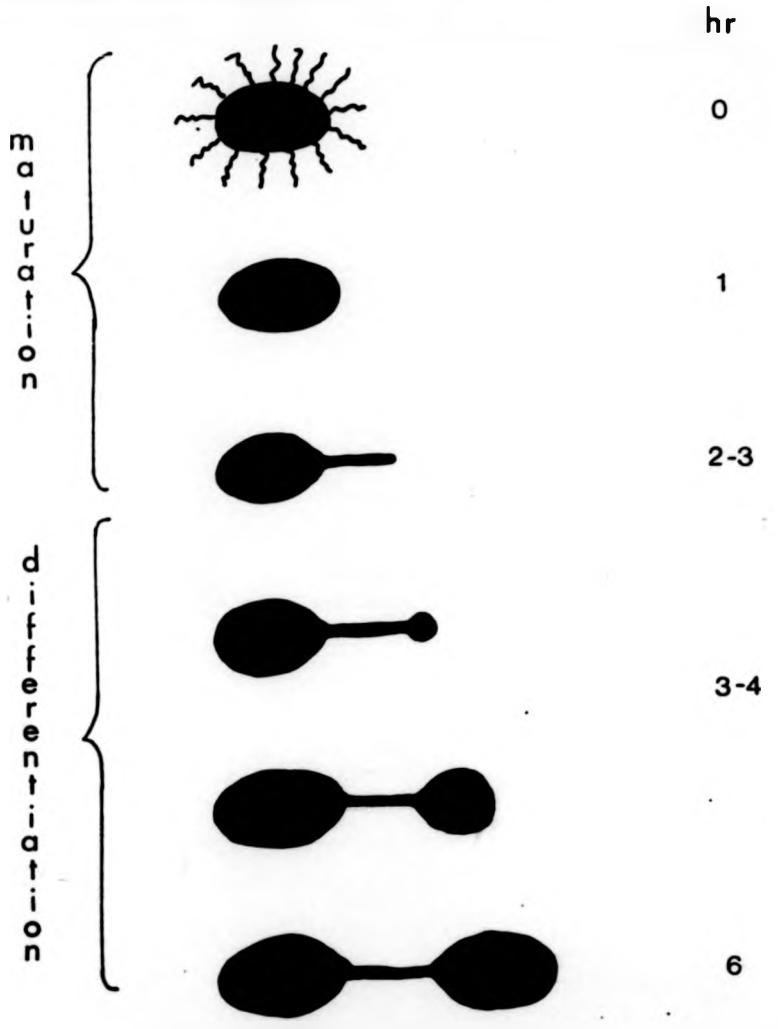


Fig. 1.17. Swarm cell maturation and differentiation

In conditions of light and high nutrient status the swarm cell undergoes firstly a period of maturation, when the flagellae are shed. After 2-3 hours a stalk or filament is produced, and a new daughter cell develops at the distal end of the filament. A 'double cell' is complete after approximately 6 hours.

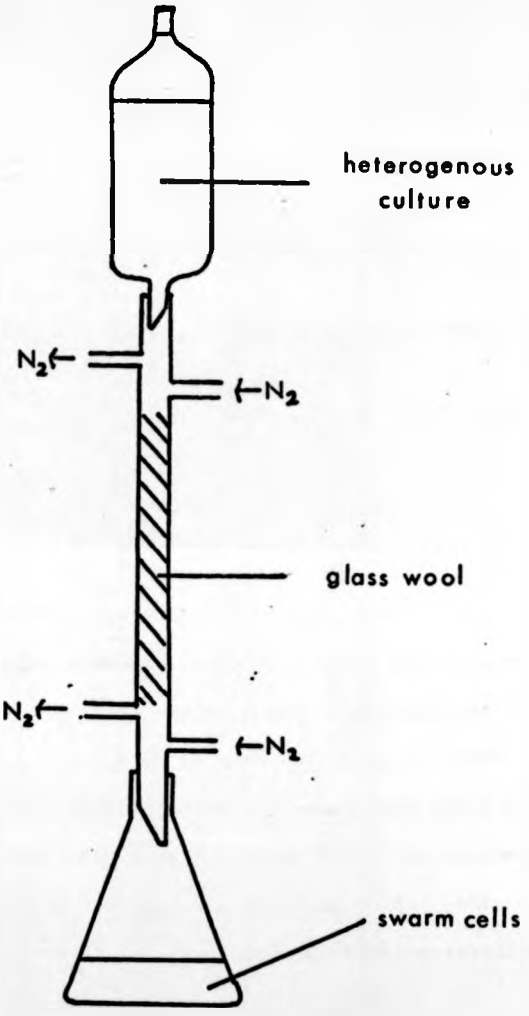


Fig. 1.18. Apparatus for the selection of homogeneous populations of swarm cells from a heterogeneous culture of R.vannielii

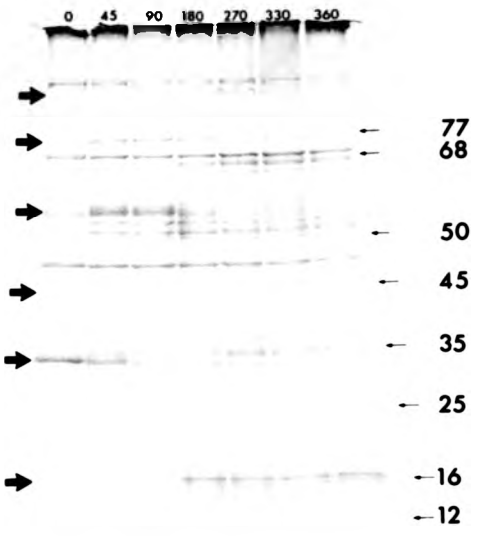
The column is flushed with nitrogen, and the glass wool is rinsed with sterile phosphate buffer prior to passing the heterogeneous culture down the column. Multicellular arrays are held back in the glass wool, whilst swarm cells pass through into the collecting flask. 200 ml - 15 litres of cells at 10^7/ml may be obtained.

Fig. 1.19. Protein synthesis during the obligate morphogenesis and differentiation sequence of R.vannielii swarm cells (Dow and France, unpublished data)

Homogeneous populations of swarm cells were selected by filtration (2:12), and at intervals aliquots were pulse-labelled with ^{35}S-methionine for 15 min. Soluble proteins were fractionated on a 10-30% (w/v) polyacrylamide gel, and the gel was autoradiographed.

The numbers at the head of each track represent the time (min) after swarm cell selection when the proteins were pulse labelled. Numbers on the right represent molecular weight markers (kilodaltons).

Heavy arrows indicate proteins which appear or disappear during the differentiation sequence.



synchronous culture
^{35}S-methionine pulse

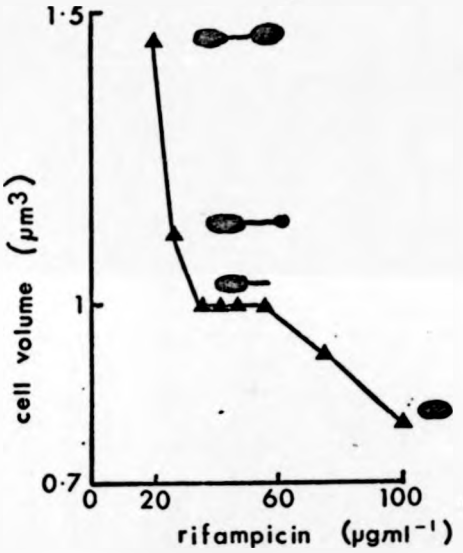


Fig. 1.20. Effect of varying rifampicin concentrations on the differentiation sequence of R.vannielii swarm cells*

Swarm cells were incubated with varying concentrations of rifampicin, and after 15 hours the morphology and cell volume distribution of the cultures were examined.

* From Whittenbury and Dow (1977)

cell differentiation. *In vitro* studies of the RNA polymerases from swarm and stalked cells has shown that they are differentially sensitive to rifampicin (Dow and Bennett, 1978). Purification and characterization of the RNA polymerases will be necessary before any further conclusions can be drawn, but the possibility of proteolysis during isolation, as encountered in *B.subtilis*, must be borne in mind.

1.7.3. *Aims of this study*. *R.vannielii* is a useful organism for studies of cellular morphogenesis and differentiation since the differentiation sequence is obligate and well-defined, and homogeneous populations of swarm cells may be obtained in large quantities. A disadvantage of the system is that the genetic and biochemical knowledge of the organism is slight, compared to some of the bacteria mentioned earlier in the review.

The aim of this study was therefore to broaden knowledge of the molecular biology of *R.vannielii*, so that further progress in the understanding of the regulatory mechanisms of differentiation can be made. It is clear from the literature review, that there are many different experimental approaches available to examine the control of differentiation. The methods used in this investigation are mainly those which have been applied to other systems, and which often required considerable modification for adaptation to *R.vannielii*.

Initially experiments were directed towards elucidation of the role of the *R.vannielii* genome, through differential gene expression, in morphogenesis and differentiation. Later this approach was broadened to include a study of how the genome might be manipulated to enable one to answer questions about the control of differentiation at the level of the gene. Since little information was available on the *R.vannielii* genome, it was necessary to characterize its physical and chemical

properties at the start of the investigation. This knowledge proved relevant at later stages of the study.

The role of the genome in one particular part of the whole cell cycle, was chosen for study. This part was the obligate differentiation of the motile swarm cell to the mature reproductive mother cell, and the subsequent reproduction of the mother cell (Fig. 1.15). This sequence was chosen for the reasons outlined earlier, namely the swarm cell undergoes a series of well-defined, obligate morphological changes during the sequence, homogeneous swarm cell populations can be readily obtained in large quantities, and the onset of differentiation is easily controlled. Additionally, an accurate swarm cell count can be made, whereas this is not possible for microcolonies of cells. Data were already available on protein and chlorophyll synthesis during swarm cell differentiation. The kinetics of macromolecular synthesis can be correlated with easily recognisable morphological events in the cell cycle.

As the study progressed, it became apparent that the genetic background must be developed to complement the biochemical data. Previous workers had attempted to obtain stable auxotrophic mutants, but had been unsuccessful, and furthermore the search for an R.vannielii phage had proved negative. Consequently, attempts were made in this study to isolate native plasmids, and conjugation experiments were initiated using the promiscuous plasmid R.68.45. Direct analysis of the R.vannielii genome was approached by the use of restriction enzymes, and specific gene coding regions were tentatively assigned to particular DNA fragments after enzyme digestion.

CHAPTER 2

Materials and Methods

2.1. Organisms

Rhodomicrobium vannielii strain Rad5 (Whittenbury and Dow, 1977) was used throughout this investigation.

Escherichia coli K12 was used in the renaturation experiments. E.coli C. met^- [R.68 45] and E.coli HB101 Str^R were used in the conjugation experiments. E.coli HB101 F^+ and E.coli HB101 F^- were used as controls in plasmid isolation experiments.

2.2. Media

a) PM medium contained per litre:-

1.5 g sodium hydrogen malate

1.5 g sodium pyruvate

0.5 g NH_4Cl

0.4 g $MgSO_4 . 7 H_2O$

0.05 g $CaCl_2 . 2 H_2O$

0.05 g NaCl

The pH was adjusted to 6.8-6.9 with KOH pellets. The medium was autoclaved at 121°C for 15 min. On cooling, 50 ml $litre^{-1}$ sterile 0.1 M phosphate buffer (0.05 M $NaH_2PO_4 . 2H_2O$, 0.05 M Na_2HPO_4, pH 6.9) was added aseptically.

b) L-broth contained per litre:-

10 g tryptone

5 g yeast extract

1 g glucose

8 g NaCl

0.22 g $CaCl . 2H_2O$

The medium was autoclaved at 121°C for 15 min.

For solid media, 17 g $litre^{-1}$ Difco bacto-agar was added prior to sterilization.

2.3. General buffers

a) Lysis buffer

0.1 M Tris-HCl, pH 7.5

10 mM EDTA

b) Resuspension buffer

10 mM Tris-HCl, pH 7.4

1 mM EDTA

c) Phenol-chloroform mixture

Redistilled phenol was mixed with chloroform and isoamyl alcohol in the ratio 25:24:1 (v/v). The mixture was then saturated with SSC.

d) Standard saline citrate (SSC)

0.15 M NaCl

0.015 M tri-sodium citrate

e) TLES buffer (for sucrose gradients)

10 mM Tris-HCl, pH 7.5

100 mM lithium chloride

1 mM EDTA

0.1% SDS

f) Tris-acetate buffer (for horizontal agarose gels)

40 mM Tris-glacial acetic acid pH 8.2.

20 mM sodium acetate

2 mM EDTA

g) 'E' buffer (for vertical agarose gels)

36 mM Tris-HCl, pH 7.7

30 mM NaH_2PO_4

1 mM EDTA

2.4. Chemicals

Wherever possible, 'Analar' grade chemicals were used. Materials were obtained from the companies listed below.

Biorad Labs, Richmond, California, USA: hydroxyapatite

B.D.H. Chemicals Ltd, Poole, Dorset, UK: caesium chloride for ultra-centrifugation, caesium chloride analytical grade

Koch Light Labs Ltd., Colnbrook, Bucks, UK: sodium N-lauroylsarcosinate

Kodak Ltd, London, UK: Xomat-H X-ray film

Nuclear Enterprises (GB) Ltd., Edinburgh, UK: 2,5 diphenyloxazole (PPO); 1,4-bis-(5-phenyloxazole-2-yl)benzene (POPOP)

Sigma Chemical Co. Ltd., Poole, Dorset, UK: Adenosine, Agarose type II, ampicillin, chloramphenicol, Kanomycin sulphate, nalidixic acid, streptomycin sulphate, RNase-free sucrose, tetracycline, 3'deoxyadenosine-5'-triphosphate, 2'-deoxycytidine-5'-triphosphate, 2-deoxyguanosine-5'-triphosphate, thymidine-5'-triphosphate, p-chloromethylphenylsulphate, n-ethylmaleimide

Whatman Ltd., Kent, UK: GF/C glass fiber discs, 3 MM filter paper

L-threo chloramphenicol was a gift from R.J. Ellis, University of Warwick.

2.5. Enzymes

Biolabs Ltd., Beverley, Mass. USA, Restriction endonucleases EcoRl. Hind III, Bam Hl

PL Chemicals Ltd, Milwaukee, Wisconsin, USA: polynucleotide kinase

Sigma Chemical Co. Ltd.,: proteinase K, RNase A, RNase Tl

Uniscience Ltd., Cambridge, UK: Sl nuclease

Worthington Biochemical Corp. New Jersey, USA: alkaline phosphatase

2.6. Radiochemicals

These were obtained from the Radiochemical Centre, Amersham, Bucks, UK:

[2-^{3}H]-adenine (15-25 Ci/mmol)

[2-^{3}H]-adenosine (20-25 Ci/mmol)

adenosine 5'-[γ-^{32}P]-triphosphate, triethylammonium salt (1000-2000 Ci/mmol)

deoxy [G-^{3}H]-adenosine (10 20 Ci/mmol)

[^{32}P]-orthophosphate in dilute HCl solution (30-100 Ci/mg phosphorus)

L-[^{35}S] methionine (200-300 Ci/mmol)

2.7. Antibiotics

Chloramphenicol, kanomycin, and streptomycin were dissolved in water and filter-sterilized, while ampicillin and nalidixic acid were dissolved in 0.01 M NaOH and filter-sterilized. When these antibiotics were added to the culture medium, there was no detectable pH change. Rifampicin and tetracycline were dissolved in 50% methanol and filter-sterilized. Fresh antibiotic solutions were prepared each week.

2.8. Gases

British Oxygen Co. Ltd., London, UK: oxygen-free nitrogen

Cambrian Chemicals, Croydon, Surrey, UK: ethylene, acetylene

2.9. Maintenance of cultures

R. vannielii, E.coli HB101, E.coli K12 and E.coli C [R68.45] were each subcultured regularly on PM agar, L-broth agar and L-broth and ampicillin (100 μg ml^{-1}) agar, respectively. R.vannielii was grown in anaerobic bags (France, 1978) at 30°C under 2000 lux illumination from tungsten lamps. E.coli strains were incubated overnight at 37°C.

2.10. Culture purity

The purity of R.vannielii cultures was checked by streaking them onto nutrient agar plates, and incubating overnight both anaerobically and aerobically at 30°C. Cultures were routinely examined by phase contrast microscopy for the presence of contaminants. E.coli strains were maintained on selective agar plates to ensure purity.

2.11. Growth of organisms

R.vannielii was routinely grown in 250 ml, B19 Erlenmeyer flasks sealed with rubber serum caps. The culture volume was 100 ml, and a 1% inoculum was used, unless indicated otherwise. Each flask was flushed for 10 min with oxygen-free nitrogen via 'inlet' and 'outlet' syringe needles inserted through the cap, and then incubated on an orbital shaker (L.H. Engineering, Stoke Poges, Bucks, UK) at 30°C, and with an incident light intensity of 2000 lux.

For growth curve measurements, R.vannielii was grown in 1 cm cuvettes (volume 3.5-4 ml), which were either filled to the brim and capped with a subaseal, or partially filled, and flushed with nitrogen for 2 min. The cuvettes were incubated in a constant temperature bath with illumination, between readings.

For the large-scale preparation of nucleic acid, R.vannielii was grown in 5 l flat-bottomed culture vessels sealed with rubber serum caps and flushed with oxygen-free nitrogen for 20 min. They were incubated with stirring at 30°C and with an incident light intensity of 2000 lux. When 500-700 ml synchronous swarm cells were required, R.vannielii was grown in 1-2 l Flow bottles, sealed, flushed, and incubated as described above.

E.coli K12 was grown in 2 l Erlenmeyer flasks for large scale nucleic acid preparation. The culture volume was 1 l and a 10 ml overnight culture was used as inoculum. Each flask was plugged with cotton wool and incubated overnight at 37°C on an orbital shaker. For conjugation experiments E.coli strains were grown in Universal bottles containing a 10 ml culture volume, and incubated at 37°C for 4 hours on an orbital shaker.

2.12. Selection of swarm cells

Synchronous populations of swarm cells were selected as described by Whittenbury and Dow (1977). A heterogeneous R.vannielii culture was grown to the mid-exponential phase of growth ($A_{540\ nm}$ ~2-2.5) prior to swarm cell selection. The culture was filtered through a sterile glass column containing glass wool (Fig. 1.18). The column had been washed previously with 500 ml sterile phosphate buffer (2.2), and was flushed continuously with oxygen-free nitrogen.

The microcolonies were retained in the glass wool, while the swarm cells passed through and were collected in an aluminium foil-covered flask. Concentrations of 10^7 swarm cells ml^{-1} were regularly obtained, and by changing the column diameter, different volumes of culture could be filtered.

The filtrate was then flushed with nitrogen as before, and incubated at 30°C with an incident light intensity of 2000 lux. The swarm cells showed 90% synchrony as judged by phase contrast microscopy and Coulter counter analysis (2.14). Populations of 'mother cells' were obtained by growing homogeneous swarm cell populations for 2-3 generations, that is about 24 hours.

2.13. Microscopy.

2.13.1. Light microscopy. Phase contrast microscopy was done using an Olympus EMT microscope, and photomicrographs were obtained with a PMT camera unit and Kodak Panatomic-X film (ASA 32). Films were developed in Kodak D19 (3 min at 20°C) and fixed in Kodafix.

2.13.2. Transmission electron microscopy. This was done using either an AEI Corinth 275 (accelerating voltage 60 Kv), or a Joel 100S electron microscope (accelerating voltage 60-80 Kv). Electron

micrographs obtained with the AEI Corinth were recorded on 70 mm Ilford line film N4E50, developed in Ilford Phenisol (1 pt + 3 pt H_2O; 4 min at 20°C) and fixed in Kodafix. Those from the Joel were recorded on Kodak electron image film 4463, developed in Kodak D19, and fixed in Kodafix.

2.14. Coulter counter analysis

Cell counts and volume analysis were done with the aid of a model ZB1 Coulter counter (Coulter Electronics Ltd, Dunstable, Beds), fitted with a Coulter Channelyser C1000 and XY Recorder II. Each sample was diluted in a known volume of 'Isoton' (Coulter Electronics Ltd) which had been filtered through a 0.22 µm filter (Millipore Ltd).

Total particle counts were made with either a 20 µm or 30 µm orifice tube. Frequency distributions of particle volumes were obtained from the Channelyser. Particle counts were expressed as counts ml^{-1}, and volumes were converted to μm^3 by the formula:-

$$V\ (\mu m^3) = \left[(\text{channel no.} \times \frac{\text{window width}}{100}) + \text{B.C.T.}\right] \times T_f$$

where B.C.T. = base channel threshold

T_f = threshold factor

Latex particles of 0.807 µm and 1.15 µm diameter were used as standards.

2.15. Spectrophotometry

Measurement of culture absorbance ($A_{540\ nm}$) and protein determinations were done in a Pye-Unicam SP500 spectrophotometer. Cuvette culture absorbances ($A_{540\ nm}$) were measured in a Pye-Unicam SP 1800 recording spectrophotometer fitted with a constant temperature cuvette housing. Absorbance of nucleic acid preparations (260 nm) were determined in a Pye-Unicam SP800A.

2.16. Protein determination

The protein concentration of cell-free extracts was determined by the method of Lowry et al., (1951). For whole cell determinations,

cells were treated with 1 M NaOH at 100°C for 5 min, before the concentration of protein was determined as above. Dried crystalline bovine plasma albumin (BSA) was used as a standard.

For a crude approximation of protein concentration, absorbance at 280 nm was measured.

2.17. Nucleic acid determination

The DNA content of whole cells was determined by the diphenylamine method of Burton (1956), and calf thymus DNA was used as a standard. RNA and DNA concentrations were measured by absorbance at 260 nm and the amount of protein contamination was estimated by measuring the absorbance at 280 nm.

2.18. Nitrogenase enzyme assay

Nitrogenase activity was assayed by the acetylene reduction method (Bergersen, 1970). A 10 ml sub-culture of R.vannielii was incubated in a 25 ml flask overnight prior to assay. 1 ml air was removed and replaced with 1 ml acetylene. Gas samples (100 µl) were removed immediately and at suitable time intervals for up to 40 mins, and analysed using a Pye-Unicam GCV chromatograph with a flame ionisation detector, a 1.6 cm x 6 mm column of Poropak R at 60°C, and a nitrogen carrier gas flow of 40 ml min^{-1}. The amount of ethylene formed was calculated from a standard curve constructed by using suitably diluted ethylene. The height of the acetylene peak on injection was monitored as an internal standard on the volume of gas injected.

2.19. Liquid scintillation counting

Radioactivity was counted on a Packard Tri-Carb Liquid Scintillation Spectrometer model 3320. The scintillation fluid used throughout contained:-

toluene	6 l
Triton-X-100	3 l
PPO	36 g
POPOP	0.45 g

10 ml of scintillation fluid was completely miscible with 1 ml of water.

The following average counting efficiencies were determined:-

a) ^{32}P in liquid 45%

b) ^{32}P on filters 44%

c) ^{3}H in liquid 29%

d) ^{3}H on filters 24%

e) ^{35}S in liquid 48%

f) ^{35}S on filters 46%

2.20. Lysis of cells

R.vannielii cells were harvested by centrifugation at 10,000 g for 15 min, and resuspended in lysis buffer (2.3). Cells were incubated at 37°C with 1 mg ml^{-1} of lysozyme for 30 min, and lysed in 1% (w/v) sodium dodecyl sulphate (SDS) at 65°C for 10 min, or 1% (w/v) sodium n-lauroylsarcosinate (sarkosyl) at room temperature. The former was used for DNA, and the latter for DNA or RNA.

E.coli cells were harvested by centrifugation as above and incubated with 1 mg ml^{-1} lysozyme at 37°C for 15-30 min. The cells were lysed with 1% SDS at room temperature.

2.21. Purification of DNA

Three methods for the purification of DNA were used.

2.21.1. DNA for renaturation studies. Protein was removed from the cell lysate by incubation with 50 µg ml^{-1} proteinase K for 1 hour at 37°C, followed by extraction with an equal volume of the phenol-chloroform-isoamyl alcohol mixture (2.3). After centrifugation at 3,000 g for 10 min, the aqueous phase was recovered and re-extracted

twice more with the phenol mixture. The aqueous phase was then extracted twice with chloroform-isoamyl alcohol (24:1), and nucleic acid was precipitated from the aqueous phase by the addition of 2 volumes of ethanol, followed by incubation at -20°C overnight in the presence of 0.3 M NaCl.

Nucleic acid was resuspended in resuspension buffer (2.3) and incubated with 50 μg ml^{-1} RNase A (previously boiled for 3 min) at 37°C for 60 min. The DNA was re-extracted as described above, and re-precipitated with ethanol. The pellet was resuspended in resuspension buffer, sheared to a size of 10^6 daltons by passage through a 27G syringe needle, and further purified by absorption to hydroxyapatite (Britten et al., 1974). The DNA was eluted from the hydroxyapatite column in 0.3 M phosphate buffer (equimolar Na_2HPO_4/NaH_2PO_4, pH 7.0).

2.21.2. Preparation of high molecular weight DNA for restriction endonuclease analysis. The DNA was phenol extracted by the method described above and dialysed against 10 mM Tris-HCl, pH 8 containing 1 mM EDTA, for 3 hours before ethanol precipitation overnight at -20°C in the presence of 0.3 M NaCl. The resultant pellet was resuspended in one tenth strength SSC (0.1 x SSC).

The nucleic acid was incubated with 50 μg ml^{-1} heat-treated RNase A for 1 hour at 37°C, followed by a further hour with 20 μg ml^{-1} T1 RNase. The DNA was then re-extracted and dialysed as described above. This preparation was either stored in 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA at 4°C, or further purified on a caesium chloride density gradient.

2.21.3. Density gradient centrifugation of DNA. Cleared cell lysates or phenol-extracted DNA preparations were layered onto

caesium chloride solutions of average density 1.71 g ml^{-1}, and centrifuged at 120,000 g for 36 hours at 10°C in an M.S.E. 65 high speed centrifuge. Gradients were harvested either from the bottom of the tube, or by upward displacement with an ISCO fractionator model 640, and DNA absorbance at 260 nm was monitored. Fractions containing DNA were pooled, and dialysed against 10 mM Tris-HCl pH 7.4, containing 1 mM EDTA, overnight at 4°C. DNA preparations were stored in the above buffer at 4°C under a drop of chloroform to prevent bacterial growth.

2.22. Purification of ribosomal RNA (rRNA)

All glassware used was washed in chromic acid or autoclaved. Unless stated otherwise, all procedures were carried out at 0°C. Nucleic acid was extracted from a cell lysate as described (2.21.1).

The ethanol-precipitated pellet was washed with 70% (v/v) ethanol containing 0.3 M NaCl, and resuspended in TLES buffer (2.3). Linear 5-20% (w/v) RNase-free sucrose gradients were formed in TLES buffer with an M.S.E. gradient maker and a Pharmacia P3 pump. Samples were layered gently onto the surface of the gradients, and these were centrifuged at 190,000 g for 6 hours at 4°C in the 6 x 14 swing-out rotor of an M.S.E. 65 centrifuge.

Fractionation of the gradients was achieved with an ISCO density gradient fractionator. Fractions containing 16S rRNA and 23S rRNA from several gradients were pooled and re-precipitated with 2 volumes of ethanol at -20°C overnight. Precipitated material was resuspended in TLES buffer, and re-centrifuged on 5-20% (w/v) sucrose gradients, from which the required fractions were pooled, dialysed and re-precipitated. The rRNA was stored at -20°C in TLES buffer.

2.23. Thermal denaturation and reassociation of DNA

2.23.1. DNA shearing. Ice-cooled DNA (~100 µg ml^{-1}) was sheared by sonicating for five one-minute periods at 1.5 amps using

a Dawe Soniprobe (20 khz). For electron microscope preparations DNA was sheared by three passages through a 25 G syringe needle.

2.23.2. Thermal denaturation of DNA. Unsheared DNA (~100 μg ml^{-1}) was melted in 0.12 M phosphate buffer (0.06 M Na_2HPO_4, 0.06 M NaH_2PO_4, pH 7). The melting characteristics of the sample were determined by monitoring the increase in absorbance at 260 nm, with increasing temperature, on a Gilford recording spectrophotometer fitted with a reference compensator. The DNA base composition (mol % GC) was estimated from the thermal denaturation temperature (T_m), the temperature at which the DNA is 50% denatured, using the formula

$T_m = 69.3 + 0.41$ (GC) (Mandel and Marmur, 1968).

This formula applies to DNA renatured in 0.2 M Na^+, but the Na^+ concentration used here (0.18 M) does not significantly alter the calculation (Mandel and Marmur, 1968; R.J. Avery, personal communication.

2.23.3. Thermal reassociation of DNA. Sheared DNA (100 μg ml^{-1}) was denatured at 100°C in phosphate buffer as above, and then allowed to renature at (T_m - 25)°C, that is 68°C in this instance. Decreasing absorbance at 260 nm with time was monitored in the Gilford recording spectrophotometer. The kinetics of the reaction were expressed by calculating the Cot (concentration x time [moles of nucleotides x seconds/litre]) required for ½ the DNA to reassociate ($Cot_{½}$: Britten and Kohne, 1968).

2.24. Spreading of DNA for electron microscopy

Sheared or unsheared DNA (~100 μg ml^{-1}) in 10 mM Tris-HCl, pH 7.4, containing 1 mM EDTA was spread for electron microscopy by the aqueous technique of Davis et al., (1971). Grids were coated with parlodion (3.5% (w/v) in amyl acetate), and carbon-coated for 15

seconds in the vacuum coating unit.

The hypophase, of either 0.25 M ammonium acetate or distilled water, was contained in a 9 cm petri dish. A chromic acid-washed, ammonium acetate-rinsed microscope slide was placed in the hypophase with one end resting on the edge of the petri dish. Spreading solution (50 µl), containing 1 µg ml^{-1} DNA, and 0.1 mg ml^{-1} cytochrome c in 0.5 M NH_4 acetate, 1 mM EDTA, pH 7.5, was applied slowly to the microscope slide just above the hypophase surface. The spreading solution ran down the slide, and formed a film over the surface of the hypophase. Grids were touched to the film at approximately one grids width from the interface, stained in fresh uranyl acetate (5×10^{-5} M) for 30 seconds, rinsed in 2-methylbutane for 10 seconds and then dried in air. The dried grids were then rotary shadowed with palladium-platinum wire at an angle of 10° in the vacuum coating unit.

2.25. Plasmid isolation

Attempts were made to isolate a putative native plasmid(s) from R.vannielii by two different methods.

2.25.1. A modified 'cleared lysate' procedure (Clewell and Helinski, 1969). Cells (250 ml) were harvested by centrifugation, resuspended in 0.1 M Tris-HCl,pH 8.0 containing 10 mM EDTA, and incubated with 1 mg ml^{-1} lysozyme for 1 hour at $37^{\circ}C$. Lysis was achieved by treatment with 1% (w/v) SDS for 10 min at $65^{\circ}C$, and the resultant cell lysate was centrifuged at 10,000 g for 20 min, the supernatant being retained.

This 'cleared cell lysate' was phenol-chloroform extracted, and treated with RNase (2.21). After ethanol precipitation, the DNA was resuspended in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, and analysed on 1% (w/v) agarose gels (2.29.1.).

2.25.2. A modified Currier and Nester (1976) technique for the isolation of large plasmids. This method depends on a procedure which selectively denatures chromosomal DNA, leaving covalently closed circular (CCC) DNA intact. Chromosomal DNA was sheared, denatured with alkali, and extracted with phenol. The denatured chromosomal DNA entered the phenol phase, leaving native plasmid DNA in the aqueous phase. DNA from the aqueous phase was centrifuged in a caesium chloride-ethidium bromide density gradient at 120,000 g for 60 hours at 15°C.

The published procedure (Currier and Nester, 1976) was used except that a different method for cell lysate preparation was used (2.20). The volume of cell lysate was 50 ml, and so chromosomal DNA was sheared by passing the lysate through an 18G syringe needle 6 times by application of a 1 Kg weight to the syringe. The refractive index and radioactivity of each of the gradient fractions was measured.

2.26. DNA polymerase assay

DNA polymerase activities I, II, III (Goss and Cozzarelli, 1973) were assayed by the method developed by Honjo et al., (1976) from the original procedure of Okasaki and Kornberg (1964). The assay mixture (total volume 250 µl) contained: 0.05 M Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 10 µM deoxythymidine-5-triphosphate (dTTP), deoxyadenosine-5'-triphosphate, deoxycytidine-5'-triphosphate, and deoxyguanosine-5'-triphosphate, all at 50 µM, 0.5 µCi ^3H dTTP, 25 µl 'activated' calf thymus DNA, and 25 µl cell lysate.

Calf thymus DNA was activated in a 20 ml reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 3 mM DNA, and 2 µg deoxyribonuclease I. The mixture was incubated at 37°C for 30 min, and then the reaction was stopped by heating at 77°C for 5 min. The cell lysate

was prepared as described in 2.20 with the omission of detergent. To distinguish each individual DNA polymerase activity, the sulfhydryl blocking agents p-chloromercuriphenylsulphate (pCMS) (0.4 mM), or N-ethylmaleimide (NEM) (2.0 mM), were added to the assay mixture (Kornberg and Gefter, 1972).

After incubation at 37°C for 30 min the assay mixture was chilled on ice, and 0.5 ml cold 10% (w/v) trichloroacetic acid (T.C.A.), containing 0.1 M sodium pyrophosphate was added. T.C.A.-precipitable material was filtered onto Whatman GF/C filter discs, washed with 5% (w/v) T.C.A., dried and the radioactivity was measured.

2.27. Culture sampling for the measurement of radioisotope incorporation into macromolecules

Samples were withdrawn from cultures either using a syringe, or via a tap if the culture vessel was an aspirator bottle. This section applies to the incorporation of ^{3}H-adenosine and ^{32}P-orthophosphate into nucleic acids, and to the incorporation of ^{35}S-methionine into proteins. In pulse-labelling experiments, incorporation of radioisotope was chased by the addition of buffer containing cold isotope. Where 'T.C.A.-precipitable counts' is stated, 10% (w/v) T.C.A. was used. For rapid estimates of ^{32}P-radiolabel, 'Cerenkov' counting (Clausen, 1968) without scintillant was used.

2.27.1. Incorporation into DNA. Aliquots (1-10 ml) were removed and incubated with an equal volume of 10% (w/v) T.C.A. for 1 hour at 0°C. After centrifugation at 3,000 g for 5 min, samples were resuspended in 1 M NaOH and incubated at 37°C overnight. The samples were reprecipitated with 10% (w/v) T.C.A. and filtered onto GF/C filter discs. These were washed in 5% (w/v) T.C.A. containing 50 µg ml^{-1} of the cold isotope (i.e. adenosine or sodium pyrophosphate), followed by

ethanol, and finally ethanol-ether (50:50). The discs were dried at 60°C for 1 hour, and the radioactivity was counted in 3 ml triton-toluene scintillant (2.19).

2.27.2. Incorporation into RNA. Aliquots (0.2-5 ml) were removed, incubated with an equal volume of 10% (w/v) T.C.A. at 0°C for 1 hour, and filtered onto GF/C filter discs. These were washed, dried and counted as described above. This procedure gave the total counts incorporated into nucleic acid, from which counts attributable to DNA were subtracted, leaving those due to RNA.

2.27.3. Incorporation into protein. Aliquots (1-5 ml) were removed, incubated with an equal volume of 10% (w/v) T.C.A. at 0°C for 1 hour, and filtered onto GF/C filter discs. These were washed, dried and counted as described above.

For rapid, crude estimates of radioactivity in a sample, aliquots (10-50 µl) were spotted onto small squares of Whatman 3 MM filter paper, dried and counted.

2.28. Restriction endonuclease digestion of DNA

High molecular weight DNA (2.21.2) was digested with three separate restriction endonucleases. The initial DNA concentration was usually 300-400 µg ml^{-1}. A diluted solution (2 units μl^{-1}) of each enzyme was prepared in the respective storage buffer (see below), and stored at -20°C. One unit of a restriction enzyme is the amount required to completely digest 1 µg λ DNA at 37°C in 1 hour.

Each assay mixture contained in 30-50 µl total volume:-

2-5 µg DNA in 10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA,

1-2 units restriction enzyme

3-5 µl 10 x assay buffer (see below)

The assay volume was made up with sterile water. The mixtures were

incubated at 37°C until complete digestion had occurred, based on the amount of DNA, and the number of enzyme units in the assays, and then heated at 65°C for 5 min to denature the enzyme.

If the DNA was to be analyzed on agarose gels, 5 µl (per 20 µl sample) of the following mixture was added:-

0.2% (w/v) agarose, 0.01% (w/v) bromophenol blue, made up in 20 mM EDTA, pH 7.5, 10 mM Tris-HCl, pH 7.8, 10% (v/v) glycerol, allowed to solidify, and then syringed through a 26G syringe needle (Schaffner et al., 1976).

Storage buffers (normal strength)

a) EcoRl (from E.coli RY13):- 0.4 M NaCl, 5 mM KPO_4 pH 7.4, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 200 µg ml^{-1} B.S.A., in 0.15% (v/v) triton-X-100 and 50% (v/v) glycerol.

b) Bam Hl (from B.amyloliquefaciens H):- 50 mM KCl, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM dithiothreitol (D.T.T.), 200 µg ml^{-1} B.S.A., in 50% (v/v) glycerol.

c) Hind III (from Haemophilus influenzae Rd cam^{-10}):- 0.05 M KCl, 0.01 M Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM D.T.T., 0.5 mg ml^{-1} B.S.A., in 50% (v/v) glycerol.

Assay buffers (normal strength)

a) EcoRl:- 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 100 µg ml^{-1} B.S.A.

b) Bam Hl:- 150 mM NaCl, 6 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 100 µg ml^{-1} B.S.A.

c) Hind III:- 0.06 M NaCl, 7 mM $MgCl_2$, 7 mM Tris-HCl, pH 7.4, 100 µg ml^{-1} B.S.A.

2.29. Agarose gel electrophoresis

2.29.1. Horizontal agarose gels. Agarose gels (0.8-1.5% w/v) were cast on a horizontal glass plate (20 x 15 cm) bounded by Sellotape to retain the agarose on the plate until it had set (Shinnick et al., 1975). The agarose was dissolved in Tris-acetate buffer (2.3), by heating to boiling point, and was allowed to cool to 60°C before it was poured onto the plate. Slot formers were 3 mm x 7 mm in size, and the gels were allowed to stabilize for 2-3 hours before the DNA was loaded.

Gels were held in a horizontal tank with a central divide and surrounded with Tris-acetate buffer in both wells up to the top surface of the gel. Buffer was pipetted into each slot before loading the samples. Samples (up to 40 µl) were loaded using an Eppendorf pipette, and electrophoresed towards the anode at 75 v for 3-4 hours, or at 20 v overnight.

2.29.2. Vertical agarose gels. Vertical gels were used to obtain better resolution of complex restriction digests. The gels were cast between two glass plates (25 x 20 cm) separated by perspex spacers (2 mm wide), and the bottom edge of the plates was sealed with several layers of insulation tape. The lower part of the gel was cast with 1.5% (w/v) agarose in 'E' buffer (2.3; Loening, 1969). The agarose mixture (20 ml) was dissolved and cooled to 83°C before pouring between the warmed plates. The separating gel consisted of 0.6% (w/v) agarose in 'E' buffer, and was poured at 65°C after the previous gel had set. The more concentrated agarose gel prevents the separating gel from slipping during electrophoresis.

When the gel had formed the insulation tape was removed and the gel was held in place with a piece of fine nylon mesh taped to the glass plates. The gels were held upright in a vertical tank with 'E'

buffer in both reservoirs. Samples (up to 60 µl) were loaded with a 100 µl Hamilton syringe, and electrophoresed at 35 v overnight.

2.29.3. Molecular weight markers for agarose gels. The following molecular weight markers were used:-

a) λ DNA digested with EcoR1. Six fragments are produced: 20.7, 7.18, 5.65, 5.27, 4.57 and 3.22 Kbp (Thomas and Davis, 1975).

b) λDNA digested with both EcoR1 and Hind III. Thirteen fragments are produced: 20.09, 5.02, 4.84, 4.19, 3.49, 2.00, 1.91, 1.58, 1.35, 0.88, 0.70, 0.50, and 0.14 Kbp (Murray and Murray, 1975).

c) 4 markers (gift of P.G. Boseley): 13.1, 8.78, 6.63, and 4.54 Kbp.

2.29.4. Staining and photography of agarose gels. DNA in agarose gels was stained for 10 min with a solution of 5 µg ml^{-1} ethidium bromide in gel running buffer. The DNA was visualized by illumination on a U.V. transilluminator model C62 (U.V. Products Inc., San Gabriel, California). Photographs were taken on a Polaroid camera with a Polaroid film 665 using an orange filter, or a SMC Pentax M camera with an orange filter and Ilford HS23 Type J500 film. Film was developed in Phenisol (1 pt and 3 pts H_2O, 4 min at 20°C), and fixed in Kodafix.

2.29.5. Fluorography of gels containing ^{3}H-labelled DNA fragments. After staining, gels containing ^{3}H-labelled fragments were fixed in 5% (v/v) acetic acid for 1 hour. Dehydration of the gel was achieved over 1 hour with three changes of ethanol. The gel was immersed in ethanol containing 3% (w/v) PPO for three hours (Bonner and Laskey, 1974). After washing in water for 45 min, the gel was dried onto Whatman 3MM filter paper at 50°C under vacuum.

2.29.6. Transfer of DNA fragments from agarose gels to nitrocellulose filters. DNA fragments were transferred to nitrocellulose filters by the method of Southern (1975) with minor modifications. Strips of gel were immersed in 0.5 M NaOH containing 1.5 M NaCl for 15 min, followed by 0.5 M Tris-HCl pH 7.0 containing 3 M NaCl for 15 min. A piece of cellulose nitrate paper (Millipore HAWP 00010, 0.45 µm pore size) longer than the gel strip was pre-soaked in 2 x SSC. A large piece of 3 MM filter paper was soaked in 20 x SSC and laid on a glass plate inside a large shallow tray (Fig. 2.1). All air bubbles were excluded from underneath the paper. The gel strip and the nitrocellulose filter were laid over the 3 MM filter paper as indicated in the figure, and the whole gel was surrounded by 20 x SSC. Transfer of the fragments was allowed to proceed overnight.

After marking the position of the gel, the filter was removed, washed in 2 x SSC for 20 min, and baked at 80°C for 2 hours. The dried filter may then be stored indefinitely until required.

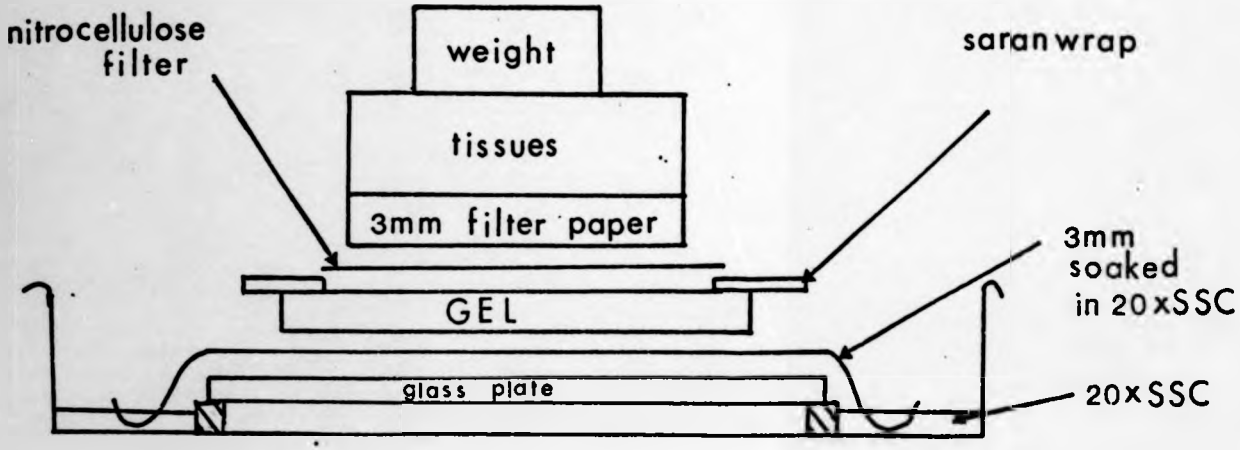
2.30. 5' end labelling of RNA

16S or 23S rRNA was resuspended in 200 µl 0.5 M Tris pH 8.0 and incubated with 5 µl alkaline phosphatase (60 units ml^{-1}) for 30 min at 37°C, to remove the terminal phosphate groups from the RNA. 10% (w/v) T.C.A. neutralized with NaOH was added to a final concentration of 250 mM, followed by 300 µl 0.3 M NaCl in 10 mM Tris-HCl, pH 8.0 containing 1 mM EDTA. The mixture was extracted with phenol-chloroform and the RNA from the aqueous layer was ethanol-precipitated for 2 hours at -20°C.

The pellet was resuspended in 5 mM Tris-HCl, pH 7.4 containing 0.01 mM EDTA and 1 mM spermidine, and heated at 50°C for 3 min. RNA,

Fig. 2.1. Southern transfer apparatus

A large piece of Whatman 3 mm paper was soaked in 20 X SSC and laid over a glass plate in a shallow tray. All air bubbles were excluded from underneath the paper. The gel was laid over the filter paper and its edges were covered with Saranwrap to prevent flow of buffer around the gel. The nitrocellulose filter, pre-soaked in 2 X SSC was laid on the gel and covered with further layers of filter paper, and a wad of tissue paper and finally a weight was placed on top. The tray contained 20 X SSC, which was drawn up through the gel, transferring the DNA fragments onto the nitrocellulose filter. Transfer proceeded overnight.



in 50 mM Tris-HCl, pH 7.6 containing 10 mM $MgCl_2$, 0.1 M sodium fluoride, 0.125 M NaCl, 5 mM DTT and 1 mM spermidine, was incubated with 1 unit of polynucleotide kinase and 10 µCi γ ^{32}P-ATP (∿5 nmol) for 30 min at 37°C (Maizels, 1976). The mixture was extracted with phenol-chloroform and ethanol precipitated several times to remove excess γ ^{32}P-ATP.

2.31. Hybridization of radioactive rRNA to DNA fragments immobilized on nitrocellulose

2.31.1. Hybridization. Nitrocellulose filters were pre-hybridized with 0.5 mg ml^{-1} carrier (yeast) RNA in 6 x SSC containing 0.5% SDS, at 70°C overnight. The filters were removed from the bags, washed in 2 x SSC four times and then incubated with 20 µg ml^{-1} RNase in 2 x SSC at 30°C for 45 min. After further washings in 2 x SSC, the filters were air-dried.

2.31.2. Autoradiography. All autoradiography was done with Kodak Xomat-H X-ray film and exposure times of 2 days - several weeks, depending upon the amount of radioactivity input. X-ray film for use with ^{3}H was pre-exposed to 1 ms light before autoradiography (Laskey and Mills, 1975). Dupont intensifying screens were used with ^{32}P radiolabel. Autoradiographs were developed in Kodak DX-80 developer, and fixed in Kodak FX-40.

2.32. Conjugation of E.coli [R68.45] and R.vannielii.

The efficiencies of several conjugation methods were assessed. The terms 'conjugation' and 'mating' were used interchangeably.

2.32.1. Conjugation in liquid culture. Cells were harvested when the concentration reached 10^8 cells ml^{-1}, washed, and resuspended to 10^9 cells ml^{-1}. Equivalent volumes of R.vannielii and E.coli were mixed, and the mixture was incubated for a specific time, either anaerobically or aerobically without shaking at 30°C. Serial dilutions

were made and the cells were allowed to recover on PM agar for 12 hours before plating onto selective media e.g. PM + ampicillin (100 $\mu g\ ml^{-1}$).

2.32.2. Conjugation on agar.

a) Agar mating on 9 cm dia. plates (Sistrom, 1977). Cells were mixed as described in 2.32.1., and then serial dilutions were made. Aliquots (20 µl) were spotted onto the centre of each plate, which was then incubated anaerobically or aerobically at 30°C for the desired mating time. The cells were spread with 50 µl phosphate buffer (2.2) and the plates were then overlayed with selective agar, and re-incubated anaerobically at 30°C with 2,000 lux light intensity.

b) Agar mating on 3 cm dia. plates or in Bijou bottles. Cells were mixed as described in 2.32.1., and 20 µl aliquots were incubated directly on either 3 cm dia. plates, or on agar in Bijou bottles. After the desired conjugation time the cells were harvested in 200 µl phosphate buffer (2.2), allowed to recover in PM medium for 12 hours, and then serially diluted. The cells (100 µl) were then spread directly onto selective plates and incubated anaerobically at 30°C with 2,000 lux light intensity.

2.33. Selection of antibiotic-resistant mutants of R.vannielii

2.33.1. Selection. A culture containing 10^9 cells ml^{-1} was serially diluted and plated onto selective plates containing one of the following antibiotics:-

a) rifampicin, 10-100 $\mu g\ ml^{-1}$

b) streptomycin, "

c) nalidixic acid, "

The plates were incubated anaerobically at 30°C with 2,000 lux light intensity. Colonies which arose were re-streaked onto the selective media on which they occurred. Mutant colonies were then

inoculated into a small volume of (PM + antibiotic) liquid medium and cultured for use in the conjugation experiments.

2.33.2. N.T.G. mutagenesis. The mutagen N-methyl-n^{1}nitro-N-nitrosoguanidine (N.T.G.) was used to increase the frequency of antibiotic-resistant mutants. R.vannielii cells were grown to a concentration of 10^8 cells ml^{-1}, harvested and resuspended in 'T' buffer (50 mM Tris-HCl, pH 7.6 containing 8 mM NaCl) to a concentration of 5×10^8 cells ml^{-1}. The cells were incubated with 100 µg ml^{-1} N.T.G. for 20 min at 30°C, harvested, washed in 'T' buffer and resuspended to 8×10^8 cells ml^{-1}. Serial dilutions were plated onto selective plates and incubated as above.

CHAPTER 3

Characterization of the R.vannielii Genome

3.1. Introduction

A pre-requisite for the study of the molecular basis of regulation of differentiation in R.vannielii is an understanding of the organisms' genetic complexity. Consequently the work described in this chapter is concerned with the physical characteristics of the genome, for example the size, base composition and genetic complexity of the DNA. These were determined by the use of methods which have been successfully applied to both prokaryotic and eukaryotic genomes (Britten and Kohne, 1968). Purified DNA is required for these studies, and the purification of DNA from R.vannielii is discussed before describing the biochemical analysis of the genome.

3.2. Preparation of R.vannielii DNA

3.2.1. Optimisation of the cell lysis procedure. Lysis of R.vannielii cells proved to be more difficult than, for example, the lysis of E.coli cells, since R.vannielii was found to be less susceptible to lysozyme treatment than is E.coli.

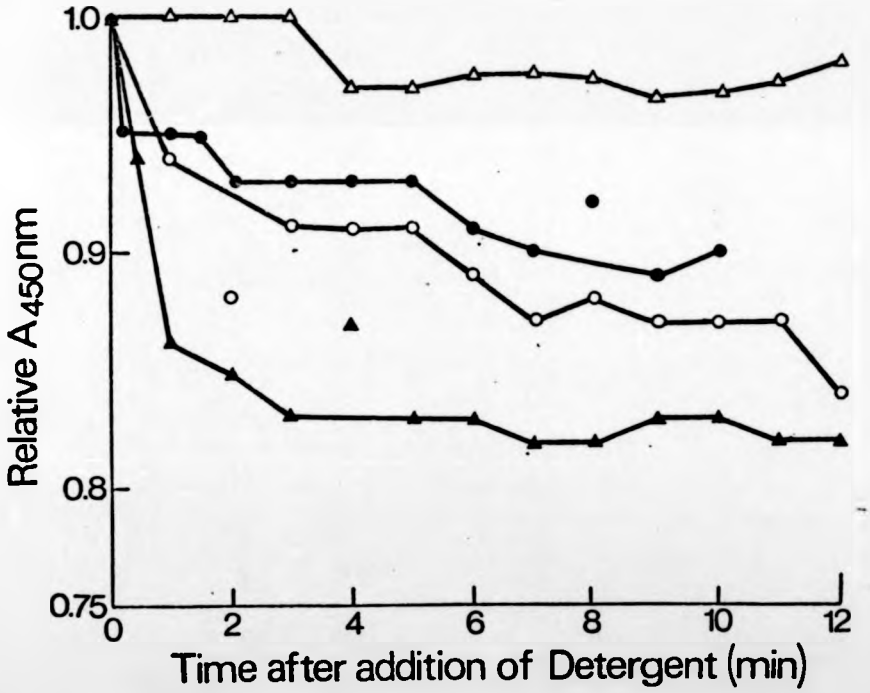
The effectiveness of several lysis procedures was examined by measuring the decrease in absorbance at 450nm (A_{450nm}) following the lysis treatment. The A_{450nm} gave a measure of the degree of cell lysis, since lysed cells cause less scattering of light than do intact cells. A young heterogeneous R.vannielii culture (A_{450nm} ~0.8-1.0) was used, ensuring that the number of clumped cells in the culture would be minimal. The results (Fig. 3.1) show that treatment with lysozyme for 30 min. was necessary for complete cell lysis, and that neither lysozyme nor detergent alone induced complete lysis. Sarkosyl treatment was slightly more effective than SDS at room temperature.

Fig. 3.1. Effect of lysis regime on the efficiency of lysis of heterogeneous R.vannielii cells

lysis regime

- ● 1 mg ml^{-1} lysozyme, 37°C, 30 min + 1% SDS
- o 1 mg ml^{-1} lysozyme, 37°C, 10 min + 1% sarkosyl
- ▲ 1 mg ml^{-1} lysozyme, 37°C, 30 min + 1% sarkosyl
- △ 1 mg ml^{-1} lysozyme, 37°C, 30 min.

An early exponential culture was treated with lysozyme for the times indicated above, and then lysed with detergent at time 0. Decreasing absorbance at 450nm (A_{450nm}) was measured with time. Relative A_{450nm} is the amount of remaining absorbance at that particular time relative to the absorbance at time 0.


1.0
0.9
0.8
0.75
Relative A_{450nm}
0
2
4
6
8
10
12
Time after addition of Detergent (min)

These results can be compared with those for E.coli (Korch et al., 1976) and C.crescentus (Evinger and Agabian, 1977) where the reduction in A_{450nm} upon lysis was much greater. The reduced efficiency of lysis in R.vannielii could be ascribed to the conformation of the cells within the microcolony, which may afford protection from the lytic agent. Another reason could be the differences in cell wall structure between R.vannielii and E.coli.

The methods of choice for cell lysis were either incubation with lysozyme (1 mg ml^{-1}) for 30 min. at 37°C, followed by lysis with 1% (w/v) sarkosyl (for RNA or DNA preparation), or incubation with lysozyme (1 mg ml^{-1}) for 30 min. at 37°C, followed by lysis using 1% (w/v) SDS and heating at 60°C for 10 min. (for DNA preparation only).

3.2.2. Purification of DNA from the cell lysate. Once lysis was achieved, the lysate was incubated with 50 µg ml^{-1} proteinase K for 1 hr. at 37°C. Protein was extracted from the lysate with an equal volume of the phenol-chloroform mixture (2.3.c) and the DNA purification procedure was as described in 2.21.1. Contamination of DNA preparations with protein was estimated by determining the absorbance of preparations at 260nm and 280nm. Most DNA samples had an $\frac{A_{260}}{280}$ of 2.7-3.0.

3.3. Thermal denaturation of DNA

DNA from all three R.vannielii cell expressions, and from E.coli K12 was denatured by heat in a recording spectrophotometer. The melting curve for each DNA was obtained by plotting the increase in A_{260nm} against time. Fig. 3.2. shows the melting curves of R.vannielii 'simplified cell cycle' DNA and E.coli DNA, while DNA from the other R.vannielii cell types gave melting curves identical to that shown for the 'simplified cell cycle' culture. The relatively low increase in absorbance (usually 36%) could be due to the presence of protein or polysaccharide in the sample.

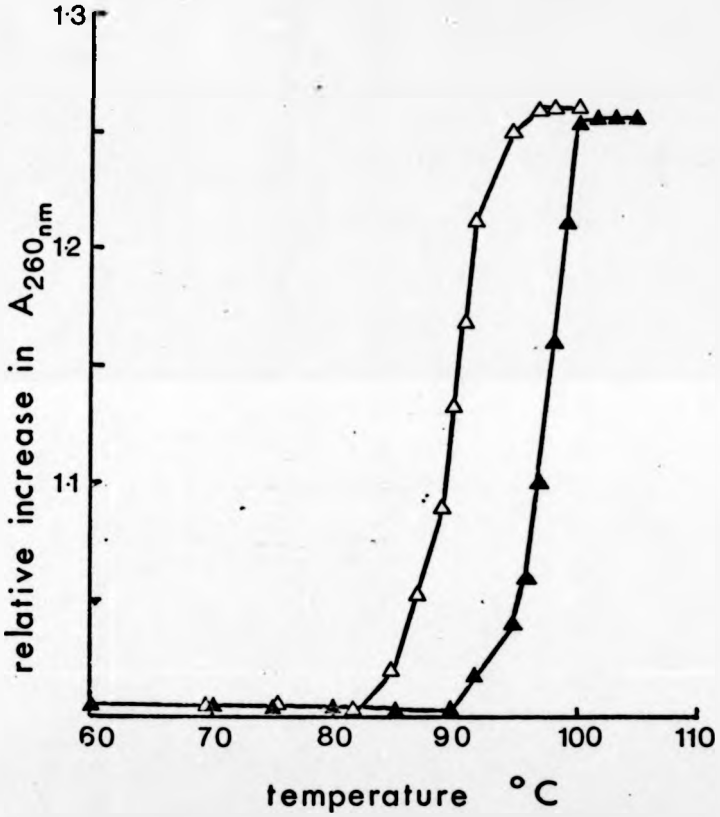


Fig. 3.2. Thermal melting profile of R.vannielii 'simplified cell cell cycle' DNA, with that of E.coli K12 DNA

▲ R.vannielii DNA

△ E.coli DNA

DNA from both R.vannielii and E.coli was isolated by phenol-chloroform extraction (2.21.2), and thermally denatured in 0.12 M phosphate buffer (2.23.2). The increase in A_{260nm} with temperature was monitored in a Gilford recording spectrophotometer.

The temperature at which half the DNA is denatured (Tm) is related to the base composition of the DNA, and therefore the mol % GC can be calculated from the formula :

$T_m = 69.3 + 0.41$ (mol % GC) (Marmur and Doty, 1962)

This formula applies when DNA is denatured in 0.2 M Na^+, and although the Na^+ concentration used here was 0.18 M, this does not affect the formula significantly (Marmur and Doty, 1962; Avery, unpublished observations). The T_m and mol % GC for each DNA are shown in Table 3.1. The average mol % GC of 62.3% compares favourably with that obtained by analytical ultracentrifugation in a caesium chloride density gradient (62.5%; Dow, 1972).

3.4. Thermal reassociation of DNA

Sonication (2.23.1) of R.vannielii DNA in phosphate buffer (2.23.2) produced fragments of approximately 1000 base pairs (bp) as determined by agarose gel electrophoresis (Fig. 3.3). The figure also shows that shearing with a syringe needle was less effective than sonication, resulting in a fragment size of 20 Kb. Molecular weights were determined by comparison with the E.coRl restriction fragments of λ DNA (Thomas and Davis, 1975, 2.29.3).

Sheared DNA was denatured (3.3) and allowed to renature at T_m-25°C i.e. 72°C, and the decrease in A_{260nm} with time was monitored in the spectrophotometer. The kinetics of the reaction were expressed by calculation of the Cot (concentration x time; moles of nucleotides x seconds $litre^{-1}$) required for ½ reaction ($Cot_{½}$; Britten and Kohne, 1968). The % single stranded DNA remaining was calculated from the hyperchromicity remaining at time t as a % of the total hyperchromicity.

In Fig. 3.4. the reassociation kinetics of R.vannielii 'mother

Table 3.1. Mean DNA base composition of R.vannielii cell expressions.

Cell type	T_m in 0.18 M Na^+ (°C)	mol % GC
R.vannielii swarm cell	95.0	62.3
R.vannielii 'mother' cell	97.2	61.8
R.vannielii 'simplified cell cycle'	97.0	62.7
E.coli	89.5	50

DNA was thermally denatured in 0.12 M phosphate buffer (2.23.2), and the T_m, the temperature at which 50% of the DNA was denatured, was measured. DNA base composition was calculated from the formula $T_m = 69.3 + 0.41$ (GC) (Marmur and Doty, 1962). T_m values are the average of three measurements.

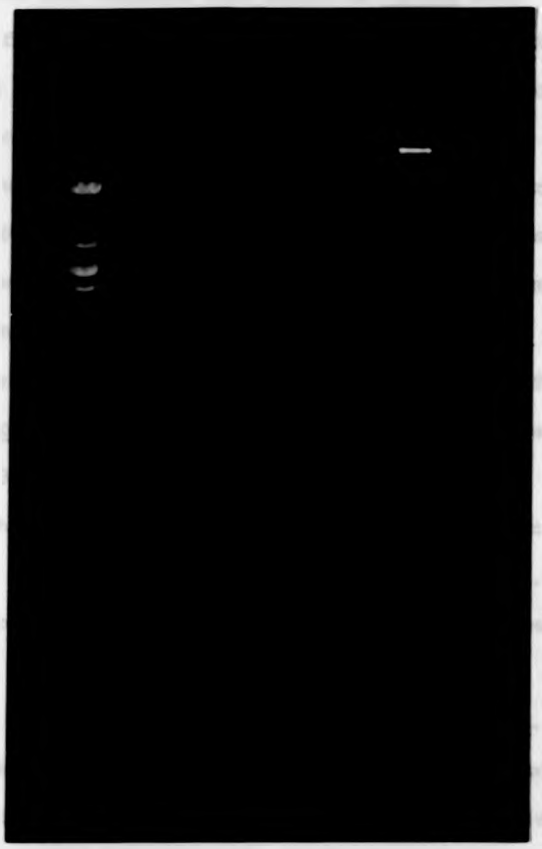


Fig. 3.3. Gel electrophoresis of sheared R.vannielii DNA

R.vannielii DNA was sheared by either sonication or passage through a 25G syringe needle (2.14), and approximately 4 µg DNA per sample was electrophoresed through 1% (w/v) agarose at 30 V overnight. The gel was stained with 0.5 µg ml^{-1} ethidium bromide and examined with U.V. illumination.

1. λ DNA restricted with EcoR1 2. sonicated R.vannielii DNA 3. needle-sheared R.vannielii DNA 4. native, unsheared R.vannielii DNA.

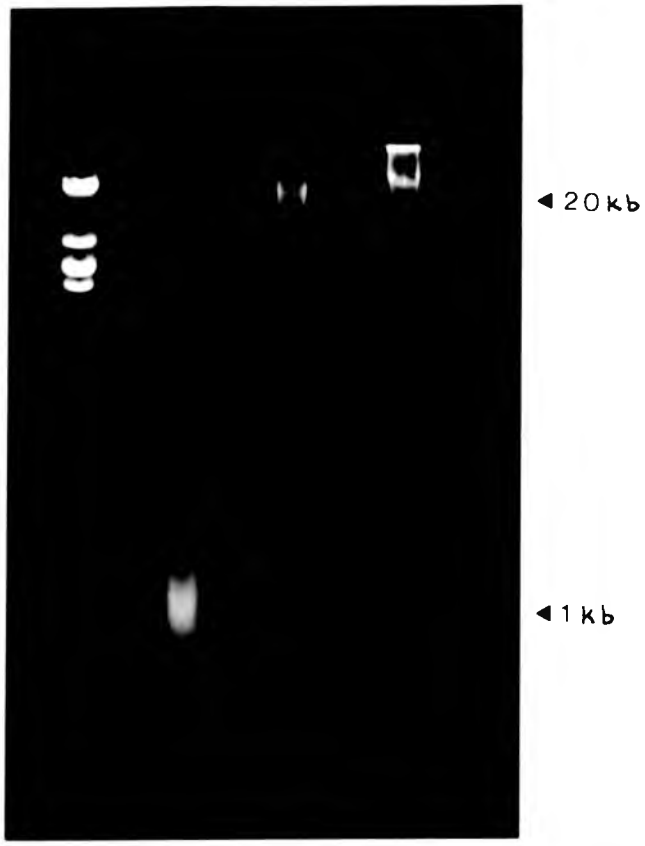


Fig. 3.3. Gel electrophoresis of sheared P.vannielii DNA

P.vannielii DNA was sheared by either sonication or passage through a 25G syringe needle (2.14), and approximately 4 µg DNA per sample was electrophoresed through 1% (w/v) agarose at 30 V overnight. The gel was stained with 0.5 µg ml^{-1} ethidium bromide and examined with U.V. illumination.

1. λ DNA restricted with PstI 2. sonicated P.vannielii DNA

3. needle-sheared P.vannielii DNA 4. native, unsheared P.vannielii DNA.

cell' DNA are compared with those of E.coli DNA. Reassociation of DNA from the other R.vannielii cell types was identical to the example shown. The bulk of R.vannielii DNA reassociated with simple second order kinetics indicating that the DNA was comprised of mainly unique sequences, and contained no large proportion of repeated sequences. In instances where the DNA is mainly comprised of unique sequences, $Cot_{\frac{1}{2}}$ is proportional to genome size (Britten and Kohne, 1968). Knowing the size of the E.coli genome (2.7×10^9 daltons; Klotz and Zimm, 1972), the $Cot_{\frac{1}{2}}$ values for R.vannielii and E.coli DNA were used to calculate the genome size of R.vannielii (Table 3.2).

One unusual feature of the reassociation data is the large initial drop at the start of renaturation (Fig. 3.4). In E.coli this 'collapse hypochromicity' represents approximately 11% of the genome, and is due to the re-stacking of bases in a non-random fashion when the temperature falls (Britten et al., 1974). This phenomenon is found in all DNA molecules, and always accounts for approximately 10% of the genome. In R.vannielii however, the total drop accounted for about 16% of the genome, suggesting that 5% of the genome was reassociating extremely rapidly. The rate of reassociation of the fraction did not vary over a 10-fold concentration range of DNA, and the renaturation may thus be due to intra-molecular reannealing.

3.5. Chemical determination of the genome size

The amount of DNA in a known number of R.vannielii swarm cells was measured by the colorimetric diphenylamine method (Burton, 1956) to confirm the estimate of genome size obtained by reassociation kinetics. A standard curve was constructed using known amounts of

Fig. 3.4. Reassociation of R.vannielii 'mother cell' DNA with that of E.coli DNA

- ● R.vannielii DNA
- o E.coli DNA

a - collapse hypochromicity

b - rapidly renaturing component

DNA from both R.vannielii and E.coli was isolated by phenol-chloroform extraction (2.21.1), resuspended in phosphate buffer (2.23.2) to 100 μg ml^{-1}, and denatured at 100°C. DNA was allowed to renature at 72°C, and the decreasing absorbance at 260nm with time was measured in a Gilford recording spectrophotometer (2.23.3).

'% single stranded DNA remaining' was calculated from the hyperchromicity remaining at time t, expressed as a % of the total hyperchromicity.

Fig. 3.4. Reassociation of R.vannielii 'mother cell' DNA with that of E.coli DNA

- ● R.vannielii DNA
- o E.coli DNA
- a - collapse hypochromicity
- b - rapidly renaturing component

DNA from both R.vannielii and E.coli was isolated by phenol-chloroform extraction (2.21.1), resuspended in phosphate buffer (2.23.2) to 100 μg ml^{-1}, and denatured at 100°C. DNA was allowed to renature at 72°C, and the decreasing absorbance at 260nm with time was measured in a Gilford recording spectrophotometer (2.23.3).

'% single stranded DNA remaining' was calculated from the hyperchromicity remaining at time t, expressed as a % of the total hyperchromicity.

Fig. 3.4. Reassociation of R.vannielii 'mother cell' DNA with that of E.coli DNA

- ● R.vannielii DNA
- O E.coli DNA
  - a - collapse hypochromicity
  - b - rapidly renaturing component

DNA from both R.vannielii and E.coli was isolated by phenol-chloroform extraction (2.21.1), resuspended in phosphate buffer (2.23.2) to 100 μg ml^{-1}, and denatured at 100°C. DNA was allowed to renature at 72°C, and the decreasing absorbance at 260nm with time was measured in a Gilford recording spectrophotometer (2.23.3).

'% single stranded DNA remaining' was calculated from the hyperchromicity remaining at time t, expressed as a % of the total hyperchromicity.

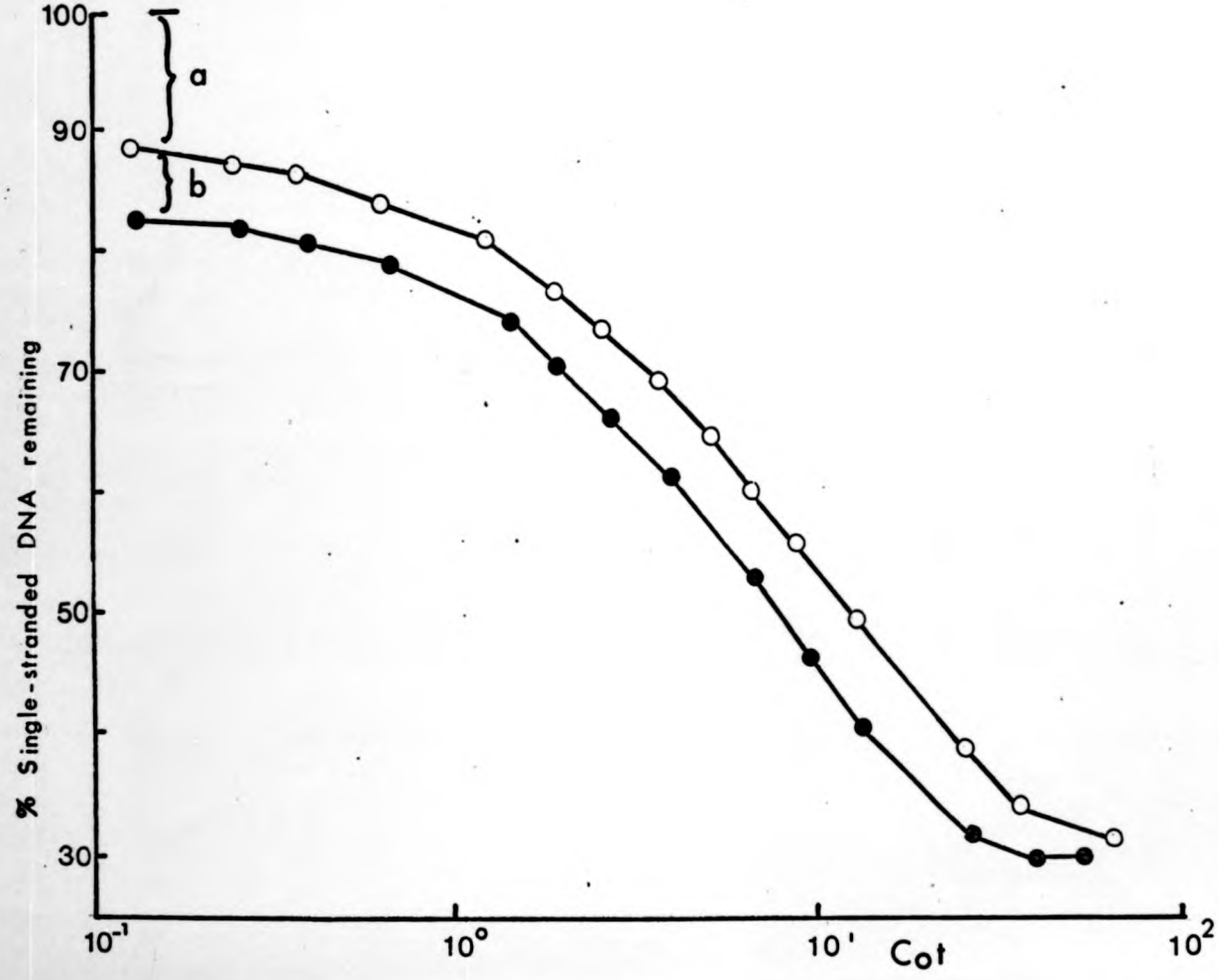

100
90
70
50
30
a
b
% Single-stranded DNA remaining
10^{-1}
10^{0}
10^{1}
10^{2}
Cot

Table 3.2. Genome size of R.vannielii cell expressions

	$Cot_{\frac{1}{2}}$	Genome size (daltons)	
		by reassociation kinetics	by the diphenylamine method
R.vannielii swarm cell	4.7	$2.16 \times 10^9 \pm 0.2$	$2.13 \times 10^9 \pm 0.3$
R.vannielii 'mother' cell	4.5	$2.10 \times 10^9 \pm 0.2$	ND
R.vannielii 'simplified cell cycle'	4.3	$1.98 \times 10^9 \pm 0.1$	ND
E.coli	6.4	2.7×10^9 [a]	

ND = no data (see 3.5) a = Klotz and Zimm (1972)

Standard errors of the means where shown, are where n = 2-4 measurements.

$Cot_{\frac{1}{2}}$ values [the Cot (concentration x time; moles of nucleotides x seconds litre^{-1}) required for ½ reaction] were calculated from the reassociation data (2.23.2). Since $Cot_{\frac{1}{2}}$ is proportional to genome size, the genome size of R.vannielii could be calculated. A second estimate was obtained by the diphenylamine colorimetric method (2.17).

calf thymus DNA. The close correlation between the two sets of data is indicated in Table 3.2. Estimation of the DNA content by the chemical method was possible only with swarm cells, since accurate counts of either 'mother cell' or 'simplified cell cycle' cell expressions are not feasible. Since no DNA replication occurs in the swarm cell (4.3.1.), the amount of DNA per swarm cell can be taken as the haploid genome size.

3.6. Attempted plasmid isolation

The fast-renaturing component in R.vannielii DNA could be due to the presence of a native plasmid. Previous attempts to isolate plasmids by analytical centrifugation followed by agarose gel electrophoresis had been unsuccessful (Dow, 1972), but two alternative methods were tried in the present study. These were the 'cleared lysate' procedure (Clewell and Helinski, 1969), and a method for the isolation of large plasmids (Currier and Nester, 1976).

3.6.1. The 'cleared lysate' procedure. Cell lysates of 1) heterogeneous R.vannielii, 2) E.coli HB101 F^+, and 3) E.coli HB101 F^- were prepared (2.25.1). The E.coli strain carrying the sex factor F was used to examine the efficiency of the method. All the lysates were phenol-chloroform extracted and the DNA was precipitated with ethanol at -20°C (2.21.2). Resuspended DNA was analysed on horizontal 1% (w/v) agarose gels electrophoresed for 4 hr. at 75 v (2.29.1). A typical gel is shown in Fig. 3.5. No plasmid was visible in the R.vannielii DNA (lanes 1 and 4), although this technique detected the F factor in E.coli HB101 F^+ (lanes 3 and 6). F is very difficult to see on the gel.

The use of a 0.4% (w/v) agarose gel also failed to detect an R.vannielii plasmid, indicating that chromosomal DNA had not

Fig. 3.5. Gel electrophoresis of cleared cell lysates from *R.vannielii* and *E.coli*

Cleared cell lysates (2.25.1) were prepared from *R.vannielii*, *E.coli* HB101F^+ and *E.coli* HB101F^- cultures. Each lysate was extracted with phenol-chloroform, and DNA from the aqueous layer was precipitated with 2 volumes of ethanol at -20°C overnight (2.21.2). DNA was resuspended in 10 mM Tris-HCl pH 7.5 containing 1 mM EDTA, and electrophoresed on a horizontal 1% (w/v) agarose gel for 4 hr at 75 V. The gel was stained with 0.5 μg ml^{-1} ethidium bromide and examined with U.V. illumination.

1 and 4 *R.vannielii* DNA

2 and 5 *E.coli* HB101 F^- DNA

3 and 6 *E.coli* HB101 F^+ DNA


1 2 3 4 5 6
'F'

masked the presence of a very large plasmid on the previous gel.

3.6.2. Method for the isolation of large plasmids. This method was developed to isolate large plasmids (10^8 daltons) from Agrobacterium tumefaciens (Currier and Nester, 1976). Heterogeneous R.vannielii cultures were labelled with ^{32}P -orthophosphate for several generations before harvesting. The principle of the method is outlined here, and the experimental details are described in 2.25.2.

Chromosomal DNA was sheared and denatured in alkali in order to enrich for plasmid DNA in the aqueous phase on phenol-chloroform extraction, since most denatured chromosomal DNA enters the phenol phase. Covalently closed circular (ccc) DNA is not denatured by alkali. The aqueous phase was fractionated on caesium chloride-ethidium bromide density gradients (2.25.2) in an MSE 65 centrifuge. Fractions from the gradient were collected, and the radioactivity in each fraction was plotted against fraction number (Fig. 3.6). A peak of radioactivity was found near the top of the gradient, while RNA was concentrated at the bottom.

Since closed circular DNA binds less ethidium bromide than chromosomal DNA, it will band at a greater density in caesium chloride than will chromosomal DNA. The peak in fraction 3 could be plasmid DNA with chromosomal contamination or chromosomal DNA itself. DNA from the peak fraction was therefore examined in the electron microscope, but no circular molecules were observed. Peak fraction DNA also failed to band as a plasmid molecule on a 1% (w/v) agarose gel. The method should have been tested using F.

It was concluded that R.vannielii did not contain extrachromosomal DNA of between 5 and 100 megadaltons in size. It is possible that a very large plasmid is present, which breaks

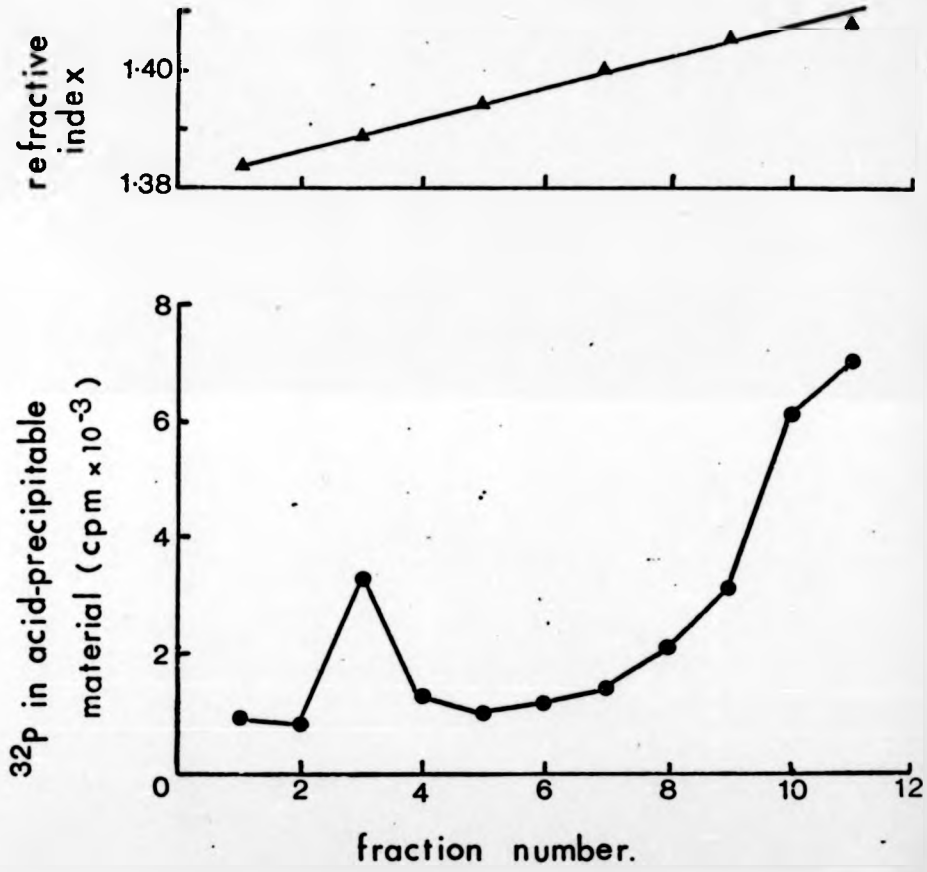


Fig. 3.6. Ethidium bromide - caesium chloride density gradient fractionation of DNA prepared by the 'Currier-Nester' technique

8 ml of the DNA sample prepared as described in 2.25.2 was mixed with 8 g CsCl and 0.6 ml ethidium bromide (10 mg ml^{-1}) in a centrifuge tube. The tubes were overlaid with liquid paraffin and centrifuged in a MSE 65 for 60 hours at 40K and 15°C. 30-drop fractions were collected from the top and 40 µl aliquots of each fraction were precipitated with 10% TCA onto GF/C filters, washed, dried and counted.

up during the extraction procedure, although it is unlikely that such a plasmid could reassociate as fast as seen in Fig. 3.4. There is as yet no genetic evidence for the existence of a plasmid in R.vannielii.

3.7. Electron microscopy of DNA

Another possible explanation of the fast renaturing component is the presence in the genome of short inverted repeat sequences, which on cooling immediately 'snap back' to form double stranded structures. Such structures should be visible in the electron microscope. To examine this possibility, needle-sheared DNA was denatured by boiling, plunged into ice to prevent reassociation, and immediately spread for electron microscopy (2.24). The molecules observed are shown in Fig. 3.7., alongside the plasmid pBR322 (MW - 2.8×10^6 daltons; Sutcliffe, 1979; derivation, Bolivar et al., 1977), which was spread under identical conditions, and used as a standard for the calibration of DNA length. The average length of the inverted repeat units was 400 bp.

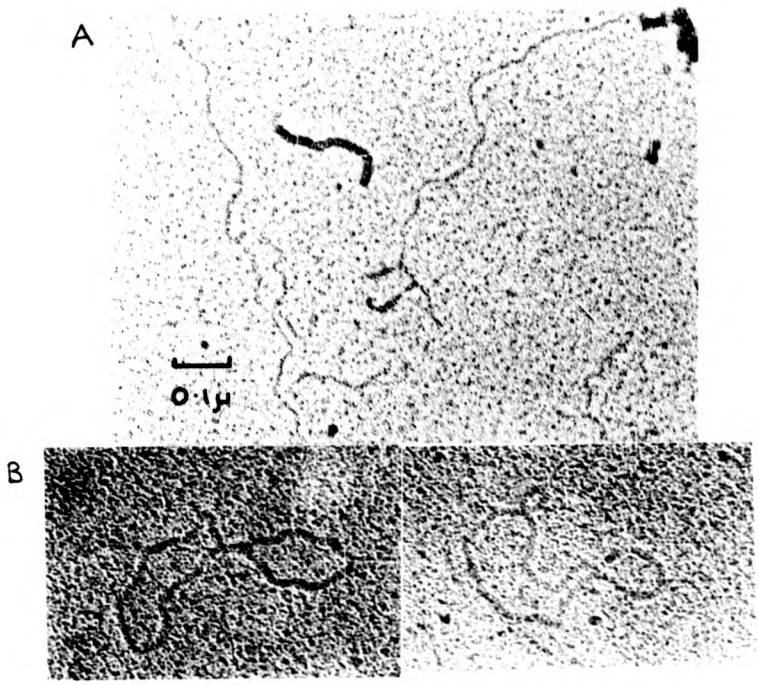
3.8. Conclusions

The experiments described in this chapter were concerned with the organization of the R.vannielii genome, by measurement of the physical characteristics of the DNA (Potts et al., 1980). In the specific areas considered, there was no significant difference between the data obtained for DNA from each of the three cellular expressions. This indicates that no large-scale gene amplification occurs during differentiation, as in some eukaryotic developmental systems (Yao et al., 1974; Wellaur and Reader, 1975; Kaback and Halvorson, 1977). Consequently the control of differentiation must operate at transcription or translation, or both.

Fig. 3.7. Electron microscopy of denatured R.vannielii DNA

R.vannielii DNA (100 μg ml^{-1}) was sheared by passage through a 25G syringe needle three times, denatured in 0.12 M phosphate buffer, and quenched on ice. DNA was spread for electron microscopy by the aqueous technique (Davis et al., 1971) (A). The plasmid pBR322, spread under identical conditions, was used for calibration (B).

DNA was treated with proteinase K (20μgml^{-1}) for 1 hour at 37°C prior to shearing.


A
0·1μ
B

The DNA base composition of R.vannielii is 62.2% GC, which compares favourably with that reported for other members of the Rhodospirillaceae (Mandel et al., 1971; Dow, 1972). The genome size of R.vannielii was found to be 2.1×10^9 daltons, which is $^2/_3$ the size of the E.coli genome.

Although smaller than that of E.coli, the genome of R.vannielii is more complex due to the presence of a rapidly renaturing component. Several explanations of this component have been considered. The rate of renaturation of the component was independent of concentration, suggesting that intramolecular rather than intermolecular hybridization was responsible.

The presence of a plasmid can be ruled out, although it may be possible to detect plasmids in freshly isolated strains of R.vannielii. Plasmids have been isolated from other Rhodospirillaceae such as R.sphaeroides (Saunders et al., 1976) and R.capsulata (Hu and Marrs, 1979), but they were not found in C.crescentus which also has a rapidly renaturing component in its DNA (Wood et al., 1976).

The electron microscopic evidence suggested that the rapidly renaturing component was due to the presence of short inverted repeat sequences dispersed along the length of the DNA. When denatured DNA was cooled, these sequences were able to 'snap back' very rapidly to form duplexes, while the rest of the molecule remained single stranded.

These sequences must be adjacent to each other, or separated by a very short piece of unique DNA, when they would appear as hairpin loops in the electron microscope. They may be a transient feature of replication, such as 'knife and fork' structures (Barzilai and

Thomas, Jr., 1970), but this possibility was excluded in R.vannielii, since the inverted repeat sequences were present in swarm cell DNA, where no replication occurs (4.3.1). The sequences had an average length of 400 bp, and represented 5% of the genome, and so it was calculated that there were approximately 200 such units per genome. Each sequence would therefore be present on average, every 16 Kb of the DNA length, if the sequences were randomly distributed throughout the genome. No data is yet available on their actual distribution.

Inverted repeat sequences have been found in both prokaryotic and eukaryotic genomes (Davidson et al., 1973; Kopecko and Cohen, 1975; Schmid et al., 1975), although their length and frequency of occurrence varies considerably. In Chlamydomonas chloroplast DNA, they represent 4-7% of the DNA and occur between 20 and 45 times per genome (Gelvin and Howell, 1979).

In C.crescentus DNA (Wood et al., 1976) inverted repeat sequences were found to occur about 300 times per genome and were between 100-600 bp in length, but no function has yet been assigned to them. Longer inverted repeats in bacteriophage Mu (Hsu and Davidson, 1974) and the Rb plasmid (Sharp et al., 1973) have been identified as insertion sequences. Sequences about 130 bp in length have been implicated in recombination between pSC50 and pSC101 (Kopecko and Cohen, 1975).

The function of the inverted repeat sequences found in R.vannielii is unknown but it is possible that they could be 'hot spots' for recombination events.

CHAPTER 4

Replication of R.vannielii DNA

4.1. Introduction

Having examined the basic organization of the R.vannielii genome, this chapter describes and discusses experiments concerned with the replication of DNA in this organism. This is of importance, since the onset of chromosome replication in R.vannielii may be a key factor in the regulation of cellular differentiation.

The study of replication throughout the whole of the dimorphic cell cycle is presently impractical, since there are no meaningful parameters, such as cell number, on which to standardize the observations. This is a consequence of the complex pattern of cellular morphogenesis and differentiation shown by R.vannielii (Fig. 1.14). However, as homogeneous populations of swarm cells can be obtained quickly and in quantity, this study considers the differentiation of the swarm cell in isolation from the rest of the cell cycle. Moreover, accurate swarm cell counts can be obtained, and the differentiation sequence is obligate and well-defined.

As measurements of DNA replication are usually based upon the temporal incorporation of radioactive DNA precursors, the selection of a suitable precursor molecule is discussed, followed by consideration of chromosome replication and of the possible role of the DNA polymerase enzymes in the control of differentiation.

4.2. Incorporation of radioactive precursors into R.vannielii DNA

In most studies of DNA replication, the incorporation of (methyl-^{3}H)-thymidine is monitored, since this isotope is preferentially incorporated into DNA, so alleviating the need to remove RNA from samples. Unfortunately, (methyl-^{3}H)-thymidine is assimilated at only a low level into R.vannielii DNA (Dow, 1972), and this is most probably due to the induction of a thymidine

phosphorylase (Bodmer and Grether, 1965). The activity of this enzyme in E.coli may be repressed by the addition of uridine (Budman and Pardee, 1967), but in R.vannielii uridine had no effect on (methyl-^{3}H)-thymidine incorporation (Dow, 1972). Previous attempts to isolate a stable thymine-requiring mutant of R.vannielii had been unsuccessful (Day, unpublished observations), and so alternative precursors were sought.

The incorporation of ^{32}P-orthophosphate into DNA with time was measured in initial experiments, but since this isotope is incorporated into nucleic acids, phospholipids and phosphoproteins, it proved difficult to eliminate radioactivity from sources other than DNA. The incorporation of ^{3}H-adenine, ^{3}H-adenosine and ^{3}H-deoxyadenosine into DNA was examined, as these isotopes will be incorporated only into nucleic acid, and radioactive RNA can be readily removed by alkaline hydrolysis.

A heterogeneous culture was incubated in the presence of ^{3}H-adenine (15-25 Ci/mmol), ^{3}H-adenosine (20-25 Ci/mmol), or ^{3}H-deoxyadenosine (10-20 Ci/mmol), until the A_{260nm} reached approximately 3.0. 5 $\mu Ci ml^{-1}$ of radioisotope were used in conjunction with 5 µM non-radioactive carrier material. Cells were harvested and lysed, and the lysate was subjected to caesium chloride density gradient centrifugation as described in 2.21.3.

The gradients were fractionated and the density profiles were determined by refractive index measurements. After dialysis against SSC, the A_{260nm} and the radioactivity of each fraction was determined (Fig. 4.1). The peaks of radioactivity in a) and c) in the figure were due to DNA since they occurred at the same position in the density gradient as would native R.vannielii DNA (buoyant density = 1.7 22$gm l^{-1}$). This was confirmed by treatment of the peak fractions with either DNase,

Fig. 4.1. Incorporation of radioactive DNA precursors into R.vannielii DNA

▲ Refractive index at 20°C

● Radioactivity (^{3}H) in acid-precipitable material (200 µl sample)

o A_{260nm}

A heterogeneous culture of R.vannielii was incubated with either a) ^{3}H-adenosine, b) ^{3}H-adenine, or c) ^{3}H-deoxyadenosine (all at 5 µCi ml^{-1}) in the presence of 5 µM of the respective cold isotope, until the A_{260nm} reached 3.0. Cells were harvested, lysed, and the resulting lysate was centrifuged in a caesium chloride density gradient to equilibrium (40K, 15°C, for 36 hours). The gradients were fractionated (1 ml fractions) from the bottom, and the refractive index, A_{260nm} and T.C.A.-precipitable radioactivity (200 µl aliquot) in each fraction was measured.

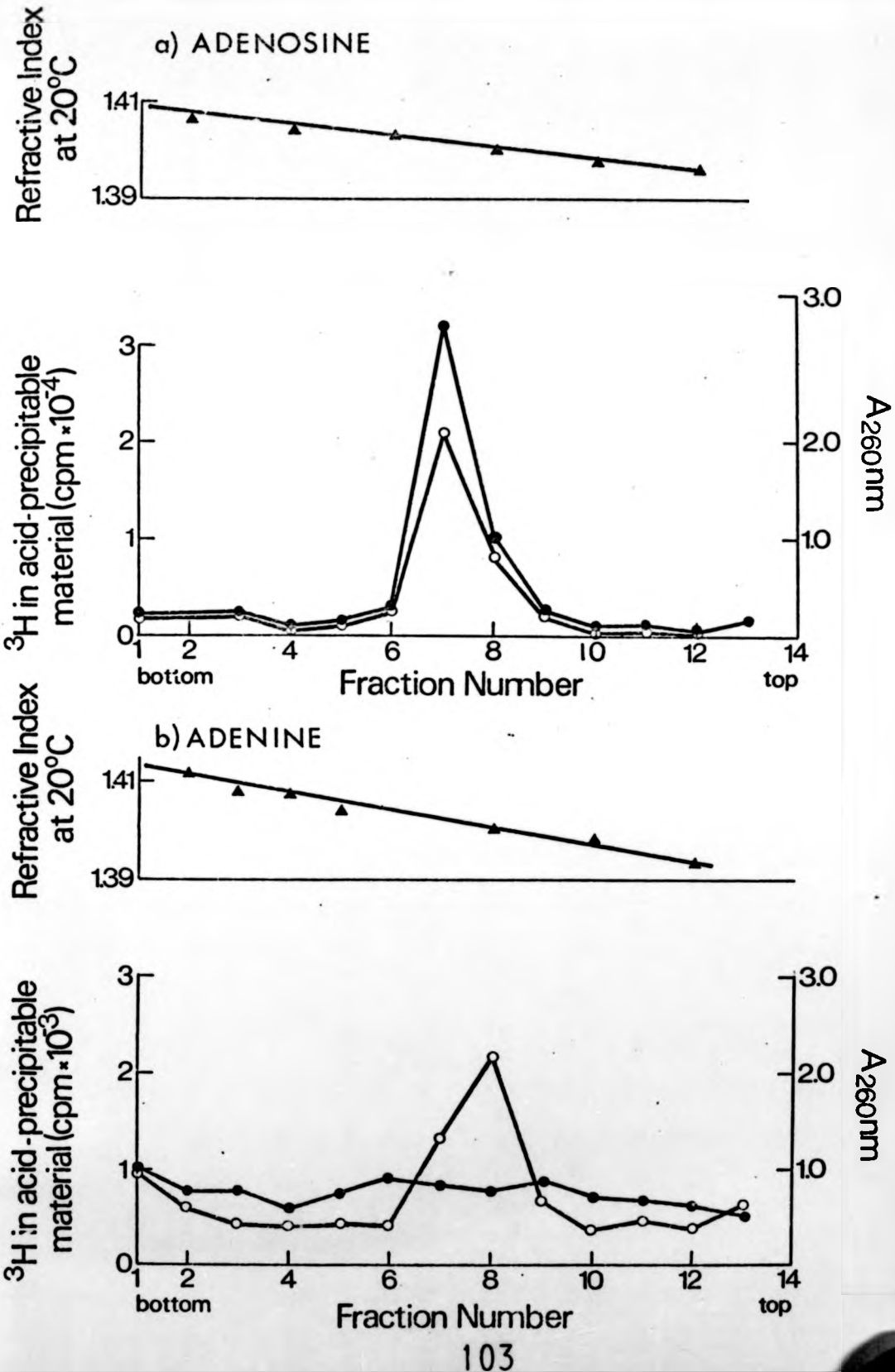
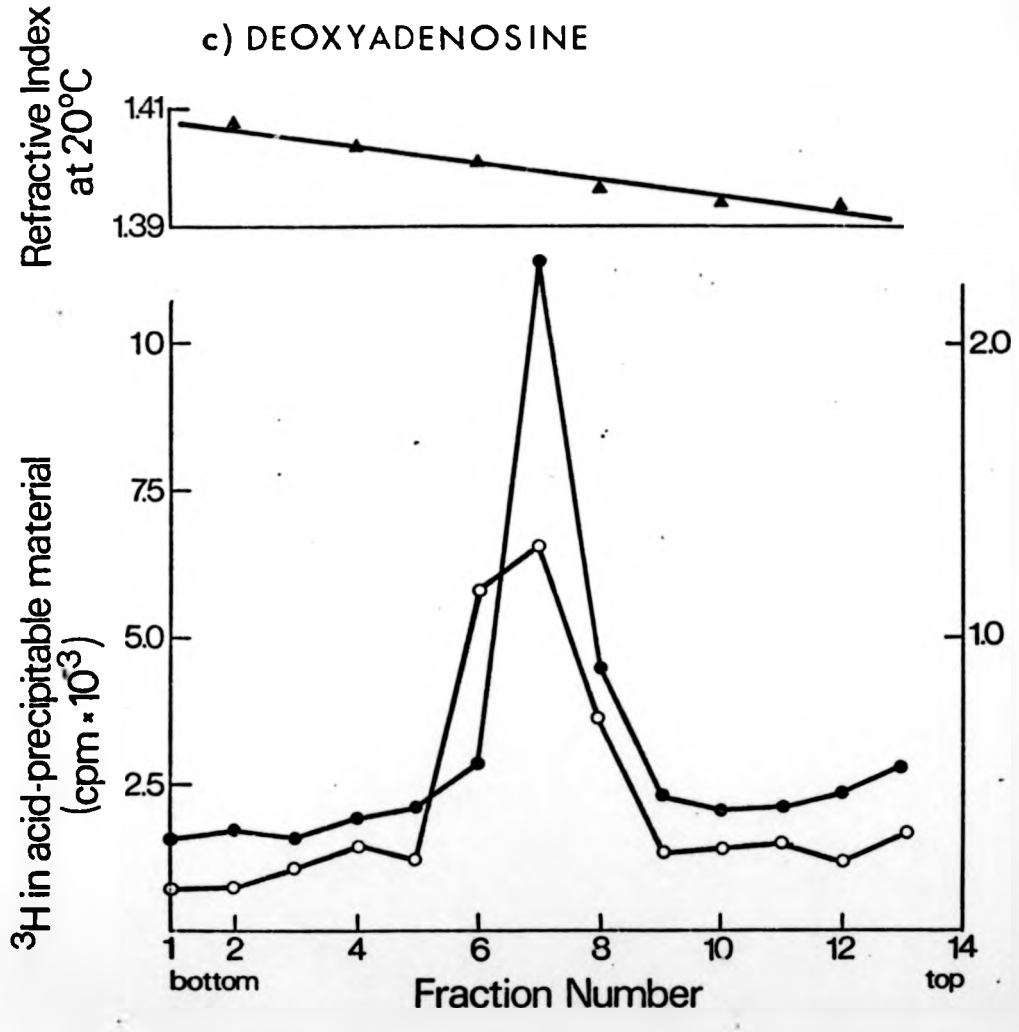

a) ADENOSINE
Refractive Index at 20°C
1.41
1.39
^{3}H in acid-precipitable material (cpm ×10^{-4})
3
2
1
0
3.0
2.0
1.0
A$_{260nm}$
1 2 4 6 8 10 12 14
bottom
Fraction Number
top
b) ADENINE
Refractive Index at 20°C
1.41
1.39
^{3}H in acid-precipitable material (cpm ×10^{-3})
3
2
1
0
3.0
2.0
1.0
A$_{260nm}$
1 2 4 6 8 10 12 14
bottom
Fraction Number
top

Fig. 4.1. continued



RNase, or both, followed by measurement of the TCA-insoluble radioactivity remaining. Most of the radioactivity was attributable to DNA, while a small amount was associated with RNA, which smeared down the gradient (Table 4.1).

It was apparent that ^{3}H-adenine was poorly incorporated into DNA, yet both ^{3}H-adenosine and ^{3}H-deoxyadenosine were incorporated efficiently, resulting in DNA with specific activities 1000 c.p.m.μg^{-1} and 2500 c.p.m.μg^{-1} respectively. Despite the more efficient incorporation of ^{3}H-deoxyadenosine, the isotope of choice was ^{3}H-adenosine, because at the levels used it showed no detectable toxic effects on the cells. Also, immediately after this experiment was done, ^{3}H-deoxyadenosine became unavailable for an extended period of time. Since ^{3}H-adenosine was incorporated into RNA as well as DNA, it was necessary to hydrolyse all samples with alkali overnight to remove RNA, prior to determining the T.C.A.-precipitable radioactivity remaining.

The effect of varying the adenosine pool size was examined, in order to optimize the conditions for incorporation of ^{3}H-adenosine into DNA (Table 4.2), and incorporation was most efficient when 5 μCi ml^{-1} ^{3}H-adenosine were used in conjunction with 5 μM cold adenosine. Higher concentrations of adenosine had increasingly toxic effects on the cells (Table 4.2). In the following experiments except where indicated, the incorporation of ^{3}H-adenosine into alkali-stable, TCA-precipitable material was used as a parameter of DNA synthesis.

4.3. DNA replication during swarm cell morphogenesis and differentiation

4.3.1. ^{32}P-orthophosphate incorporation. Initial experiments were undertaken to follow the temporal incorporation of ^{32}P-orthophosphate into DNA. A volume of between 200-300 ml of homogeneous

Table 4.1. Analysis of the peaks of radioactivity seen in Fig. 4.1. a) and c).

Gradient peak	[^{3}H] remaining in acid-precipitable material (cpm)			
	before treatment	RNase alone	DNase alone	RNase/ DNase
a) ^{3}H -adenosine	30,000	25,000	9,500	2,300
c) ^{3}H -deoxyadenosine	11,000	10,000	2,500	500

200 µl aliquots of the peak fractions were counted, and then incubated with either RNase (50 µg ml^{-1}), DNase (50 µg ml^{-1}) or both, for 1 hour at 37°C. Nucleic acid remaining was precipitated with 10% T.C.A. onto GF/C filters, washed, dried and counted. The data represent an average of three estimates.

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Table 4.2. Effect of adenosine pool size on the incorporation of ^{3}H -adenosine into DNA

Concentration of adenosine (μM)	Increase in A_{540nm} over the period of incubation	(^{3}H)in alkali-stable, acid-precipitable material (cpm x 10^{-3})
0	3.17	40.5
0.5	3.36	43.5
1.0	3.17	52.9
2.0	3.22	55.0
5.0	3.33	65.0
10	3.19	54.5
100	3.14	63.4
500	2.24	57.6

'Simplified cell cycle' cultures of R.vannielii were incubated with varying concentrations of adenosine, and 5 μCi ml^{-1} ^{3}H -adenosine for 3 days. The A_{540nm} of the cultures were measured initially and at the end of the incubation period. Cells (15 ml) were harvested, and nucleic acids were precipitated with 10% T.C.A. containing 50 μg ml^{-1} adenosine carrier. After resuspension, the samples were incubated with 1M NaOH for 15hr at 37°C, and the remaining acid-precipitable material was filtered onto GF/C filters, washed with 5% T.C.A., dried and their radioactivity counted.

swarm cells was selected (2.12), and incubated with 3 μCi ml^{-1} ^{32}P-orthophosphate (50 Ci/mg phosphorus). Each time swarm cell populations were selected, an aliquot was withdrawn for a cell count and cell volume analysis in the Coulter counter and channelyzer (2.14). The swarm cell count was always between 1-2 x 10^7/ml, and a representative cell volume profile is shown in Fig. 4.2. Coulter analysis was used to check on the uniformity of swarm cell populations.
Samples were taken at regular intervals, and total TCA-precipitable, and alkali-stable TCA-precipitable radioactivity was measured. At each time point the culture was examined microscopically, and the A_{540nm} was measured (Fig. 4.3.a).

The increase in A_{540nm} demonstrated three phases of swarm cell differentiation; in the first stage the flagellae were shed, and a small increase in A_{540nm} occurred; during the second stage (stalk synthesis), little increase in A_{540nm} took place; and in the third stage, when daughter cell formation occurred, a large increase in A_{540nm} was observed.

There was no incorporation of ^{32}P into DNA during the first three hours, but considerable incorporation took place during the 3-5 hour period thereafter (Fig. 4.3.b). This period of incorporation corresponded with the conclusion of stalk synthesis and the onset of bud formation. There was no DNA replication in the swarm cell prior to differentiation, initiation of replication occurring at or around the completion of stalk synthesis.

Further similar experiments, and several pulse-labelling experiments were undertaken using ^{32}P -orthophosphate, but the reproducibility between and within experiments was poor. Therefore the incorporation of ^{3}H-adenosine into DNA was measured in all subsequent studies concerned with the kinetics of nucleic acid synthesis.

4.3.2. ^{3}H-adenosine incorporation. Incorporation of ^{3}H-adenosine into the DNA of differentiating swarm cells (Fig. 4.4.a) followed the same pattern as the incorporation of ^{32}P -orthophosphate. There was no incorporation of radioactivity for the first two hours, followed by a significant rise in incorporation once daughter cell synthesis was initiated. The onset of DNA replication was again correlated with the completion of stalk synthesis.

Fig. 4.4.b. illustrates the incorporation of ^{3}H-adenosine into the DNA of an exponentially growing 'simplified cell cycle' culture. This served as a control since in this cellular expression asynchronous cell division occurs throughout exponential growth (France, 1978). The 'simplified cell cycle' culture was passed through a synchronization unit (Fig. 1.1B), so that it was prepared under the same conditions as the swarm cell culture. The incorporation of ^{3}H-adenosine was constant throughout the time course and did not show reduced incorporation during the first two hours, indicating that the 'lag' in incorporation shown by homogeneous swarm cell populations was peculiar to the swarm cell, and not due to physiological shock imposed by the synchronization technique.

4.3.3. Pulse labelling of swarm cell DNA with ^{3}H-adenosine. Swarm cell DNA was pulse-labelled with ^{3}H-adenosine to determine the lengths of the S and G_1 periods during the cell cycle (Fig. 4.5). The experiment was continued for 20 hours (approximately 3 generation times), and the length of the pulse label was 2 hours.

DNA replication was not discontinuous, since after the completion of the first cell division, incorporation continues to increase. Consequently it was difficult to estimate the length of the S phase. However, if one assumed a decrease in incorporation after the mid-point of DNA synthesis symmetrical with the increase, then the duration of S phase was 1½ hours, and of G_1 4-5 hours. The G_1 could be reduced to

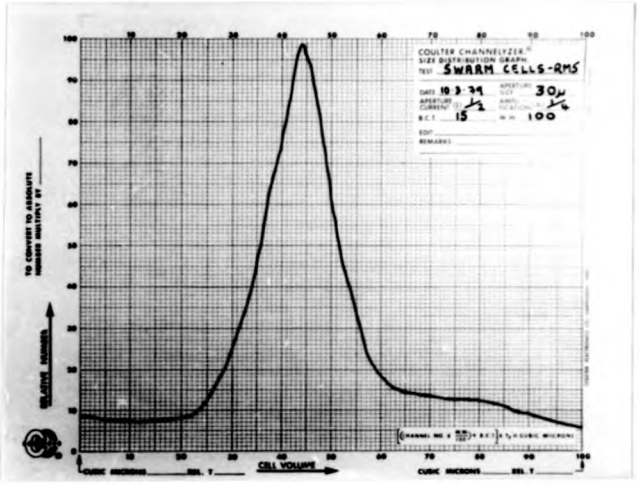


Fig. 4.2. Cell volume distribution of a homogeneous swarm cell population.

After swarm cell selection by filtration (2.12), an aliquot was diluted in Isoton, and analysed in the Coulter counter and channelyser (2.14).

Swarm cell count = 1.2×10^7 ml^{-1}

Fig. 4.3. Growth rate and DNA replication during swarm cell morphogenesis and differentiation.

A homogeneous population of swarm cells was selected by filtration (2.12), and then incubated in the presence of 3 μCi ml^{-1} ^{32}P -orthophosphate. Growth rate of the culture was measured by the increase in A_{540nm} with time. 0.1 ml aliquots were withdrawn at intervals, and incorporation was stopped by the addition of 10% T.C.A. containing 0.1 M sodium pyrophosphate. After hydrolysis in 1 M NaOH overnight at 37°C, T.C.A. precipitable material was collected on GF/C filters, which were then washed, dried and their radioactivity measured.

T.C.A. precipitable radioactivity remaining after alkaline hydrolysis is attributable to radioactivity incorporated into DNA, there being ∿ 1% of the radioactivity incorporated into macromolecules other than nucleic acids.

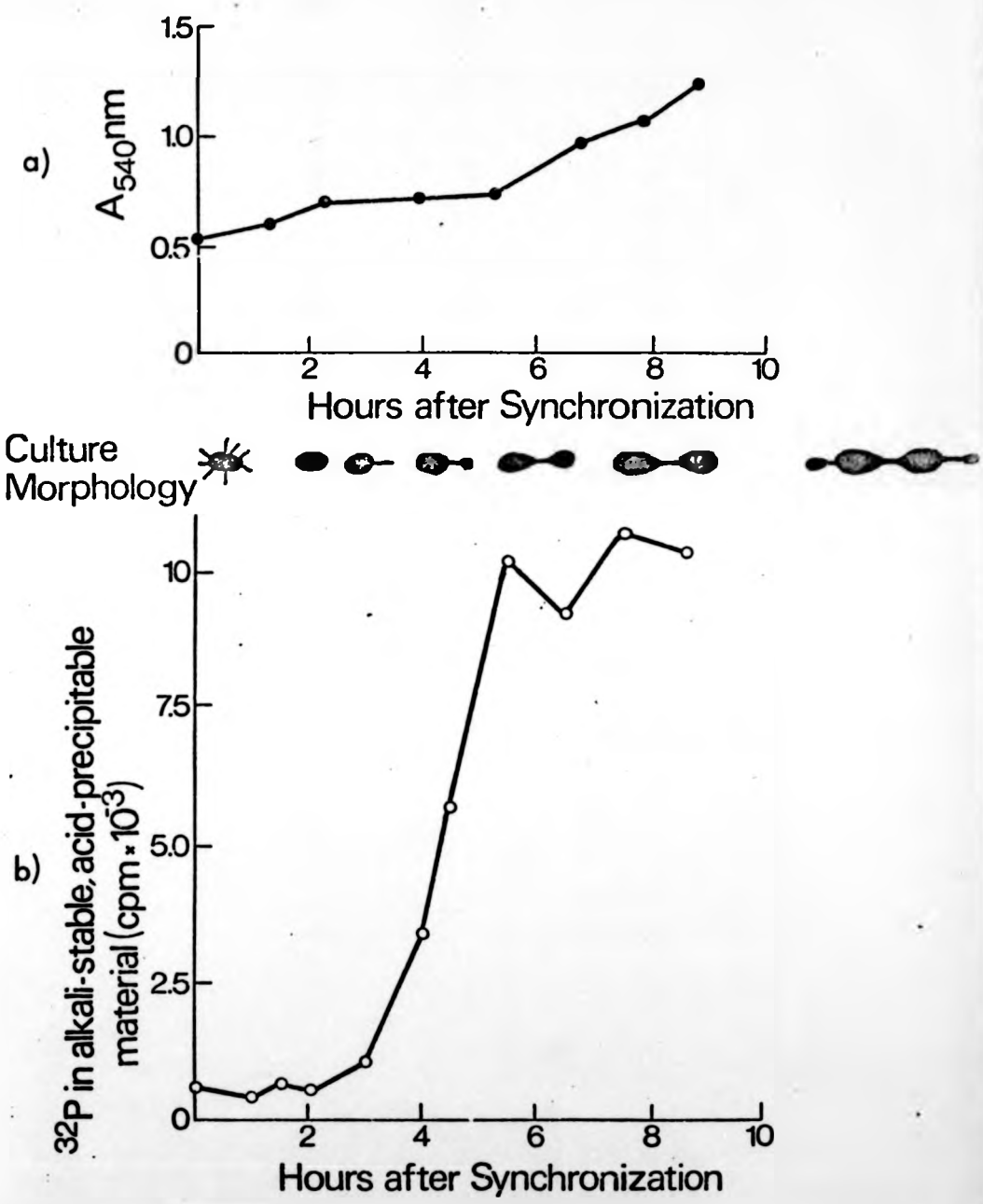
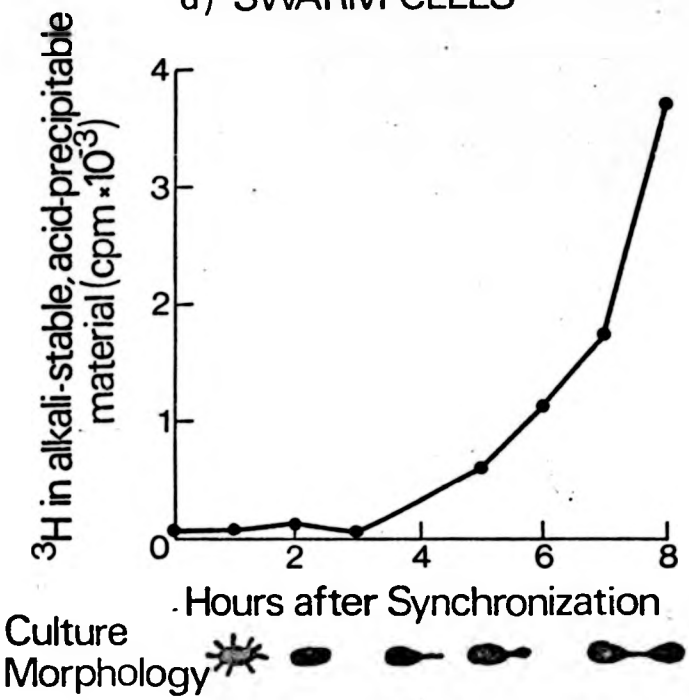

a)
A_{540nm}
1.5
1.0
0.5
0
2
4
6
8
10
Hours after Synchronization
Culture Morphology
b)
^{32}P in alkali-stable, acid-precipitable material (cpm × 10^{-3})
10
7.5
5.0
2.5
0
2
4
6
8
10
Hours after Synchronization

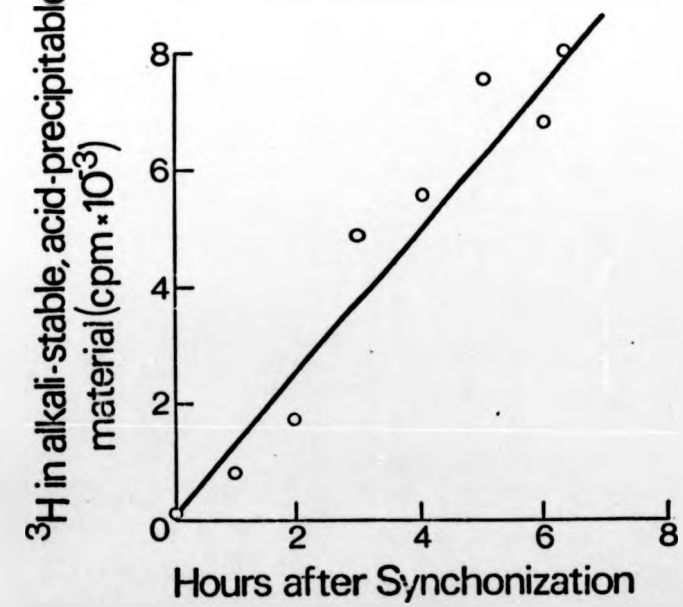
Fig. 4.4. DNA replication during swarm cell morphogenesis and differentiation, and in exponential cells from the 'simplified cell cycle'.

Homogeneous swarm cell populations (a), or early exponential cells from the 'simplified cell cycle' (b), were incubated with 10 μCi ml^{-1} ^{3}H -adenosine, and 5 μM adenosine carrier. At intervals, samples [(a) 10 ml; (b) 1 ml] were withdrawn, and incorporation was stopped by the addition of 10% T.C.A. containing 50 μg ml^{-1} adenosine. After hydrolysis in 1 M NaOH overnight at 37°C, the remaining T.C.A.-precipitable material was collected on GF/C filters, which were washed, dried and their radioactivity measured (2.27.1).

a) SWARM CELLS



b) "SIMPLIFIED CELL CYCLE"



2-3 hours, under different environmental conditions, e.g. higher light intensity, and also depending on the 'energy status' of the cell. This is a poorly understood phenomenon which may arise from the fact that when swarm cells are selected from a culture, they vary in age both within and between cultures, depending on how long they were present in the culture prior to selection. Consequently the G_1 or 'lag' period may vary.

After the first synchronous cell division, further divisions were asynchronous, and therefore it was not possible to follow the pattern of DNA replication in subsequent cell divisions. Nevertheless since incorporation did not fall after the first division, it appeared that no 'maturation period' was necessary in the stalked cell, and that DNA replication was continuous (see discussion).

No further pulse labelling experiments were done, since it was decided to pursue a different line of investigation. However other pulse labelling experiments would be of use if they employed a much shorter labelling period and more radioactive label.

4.3.4. Effect of light on nucleic acid synthesis and differentiation in the swarm cell. When a homogeneous population of swarm cells was kept in the dark, no differentiation occurred, but the swarm cells remained viable for at least 15 hours. Thus, swarm cell development was dependent on light, and consequently nucleic acid synthesis was compared between cells grown in the light and cells grown in the dark.

Homogeneous swarm cell populations were incubated in the light or the dark, and the incorporation of ^{3}H-adenosine into DNA and RNA was measured at regular intervals. A 'simplified cell cycle' culture served as a control. The incorporation of ^{3}H-adenosine into the DNA of all three populations is shown in Fig. 4.6. Swarm cells in the

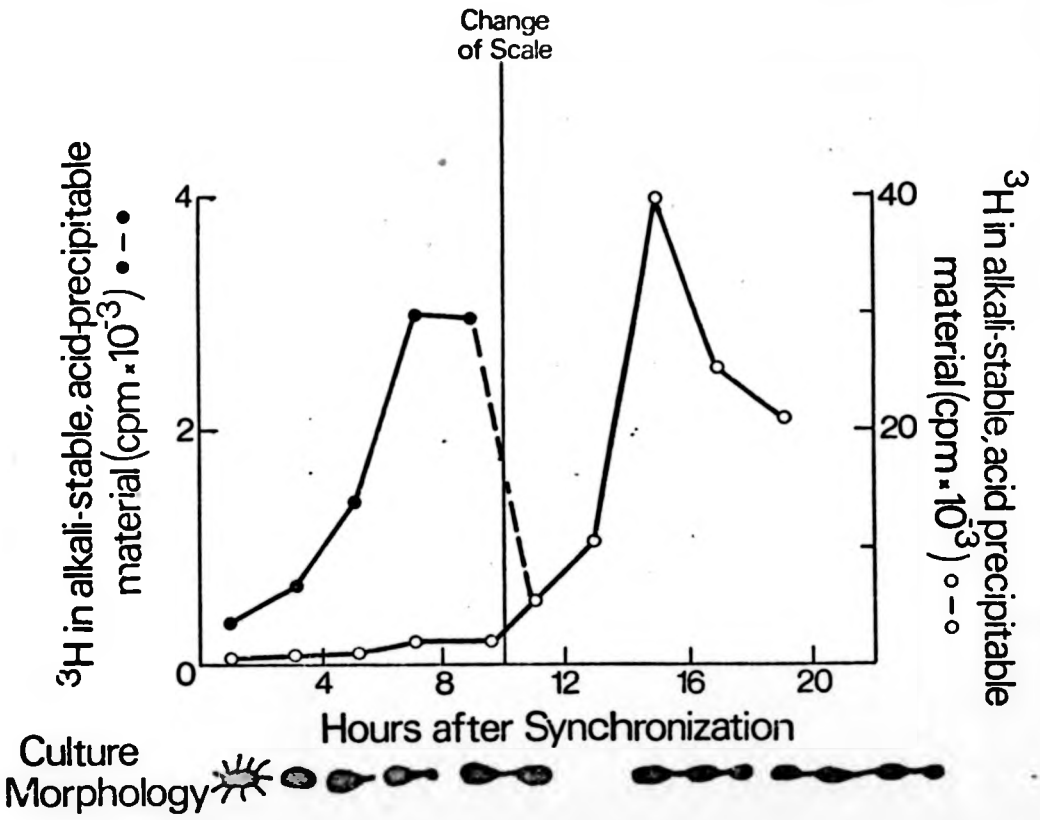


Fig. 4.5. Pulse labelling of DNA during swarm cell morphogenesis and differentiation

A homogeneous population of R.vannielii swarm cells was selected by filtration (2.12) and incubated phototrophically at 30°C. 15 ml aliquots were withdrawn at intervals and pulse labelled for 2 hours with 10 µCi ml^{-1} ^{3}H -adenosine, in the presence of 5 µM adenosine carrier. Incorporation was stopped by the addition of 10% T.C.A. containing 50 µg ml^{-1} adenosine carrier. Precipitated material was hydrolysed in 1 M NaOH overnight at 37°C, and then T.C.A.-precipitated onto GF/C filters. The filters were washed, dried and their radioactivity counted (2,27,1).

light showed the characteristic 'lag' period before the onset of DNA replication, whereas the 'simplified cell cycle' control cells did not. Swarm cells incubated in the dark however, showed little DNA synthesis. The small amount of incorporation in these cultures was due to replication in the small proportion of cells which continued to develop in the absence of light; 90% of the swarm cells remained motile in the dark.

The incorporation of ^{3}H-adenosine into total RNA is shown in Fig. 4.7. In the light-incubated swarm cells RNA synthesis proceeded continuously, with the rate increasing significantly during daughter cell synthesis. No 'lag' period was seen at the start of the experiment, as with DNA. The early RNA synthesis seen in the swarm cell may be the result of synthesis of mRNA for swarm cell-specific proteins (Fig. 1.19). In dark-incubated cells there was little RNA synthesis initially, but after 3-4 hours some synthesis did occur. This could have been due to RNA synthesis in the few cells which initiated development in the dark, or to RNA synthesis associated with maintenance functions. Swarm cells retained their ability to differentiate after 15 hours in the dark, perhaps indicating that they were able to switch to fermentative metabolism (Uffen and Wolfe, 1970).

4.4 DNA polymerase activity during swarm cell morphogenesis and differentiation

4.4.1. Introduction. DNA polymerases catalyse the addition of mononucleotides from deoxynucleoside-5'-triphosphates to the 3'-hydroxyl terminus of a DNA primer. As these enzymes have been implicated in the regulation of DNA replication in the cell (Ciarrochi et al., 1977), the activities of DNA polymerases during swarm cell morphogenesis and differentiation were investigated.

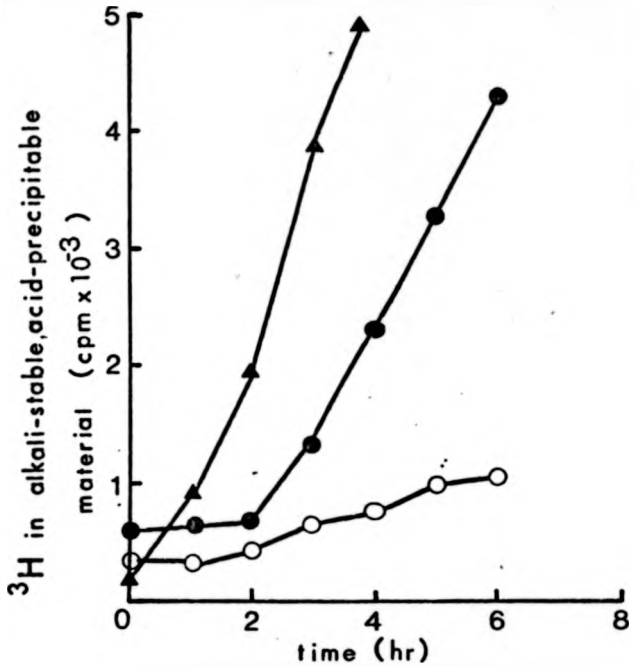


Fig. 4.6. Effect of light on DNA synthesis during swarm cell morphogenesis and differentiation.

● Homogeneous swarm cells incubated in the light

○ " " " " " " dark

▲ cells from the 'simplified cell cycle' in the light (control)

Homogeneous swarm cell populations were incubated anaerobically either in the dark or the light at 30°C, and at intervals 5 ml samples (control - 1 ml) were withdrawn, and incorporation was stopped by the addition of 5% T.C.A. containing 50 µg ml^{-1} adenosine carrier. Samples were hydrolysed in 1M NaOH overnight, and 10% T.C.A. precipitable material was collected on GF/C filters. The filters were washed, dried and counted (2.27.1).

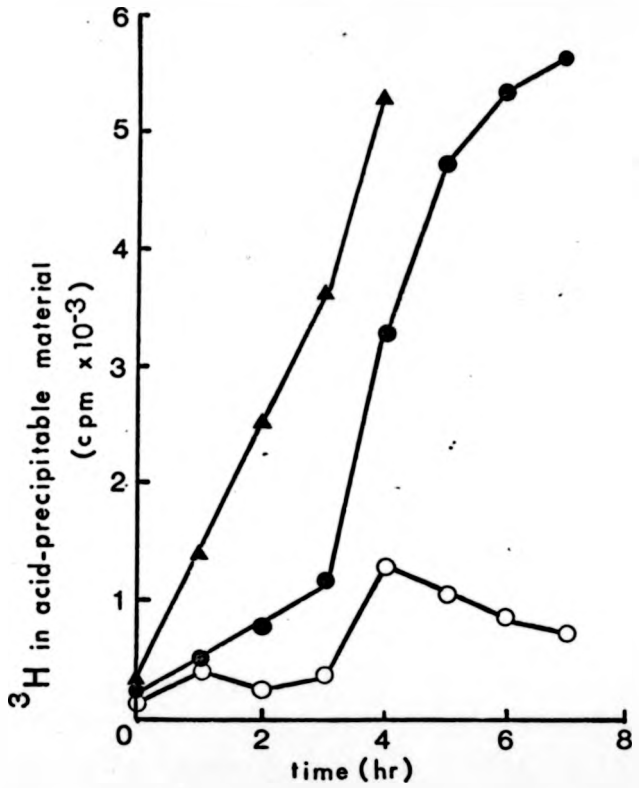


Fig. 4.7. Effect of light on RNA synthesis during swarm cell morphogenesis and differentiation

● swarm cells incubated in the light

○ " " " " " dark

▲ cells from the 'simplified cell cycle' in the light

Homogeneous swarm cell populations were incubated anaerobically either in the dark or in the light at 30°C, and at intervals 1 ml samples (control - 0.2 ml) were withdrawn and precipitated with 10% T.C.A. containing 50 µg ml^{-1} adenosine carrier. Total acid-precipitable material was filtered onto GF/C filters, washed, dried and counted (2.27.2). Counts attributable to DNA were subtracted from the total T.C.A.-precipitable counts to give the counts due to RNA.

In E.coli and B.subtilis, three DNA polymerase enzymes have been identified. DNA polymerase I is associated with repair synthesis (e.g. of UV-induced damage) and discontinuous strand replication (Kelly et al., 1969), DNA polymerase II, whose physiological role is unknown (Wickner et al., 1972), and DNA polymerase III is responsible for replication itself (Otto et al., 1973). The isolation and characterization of DNA polymerases from R.vannielii was not attempted, but DNA polymerase activity was assayed during swarm cell differentiation, exploiting the observation that individual DNA polymerase activities may be distinguished by the use of specific inhibitors (sulfhydryl blocking agents) in the assay mixture (Kornberg and Gefter, 1972).

4.4.2. Effect of primer DNA and swarm cell concentration on the efficiency of the DNA polymerase assay. Lysozyme becomes ineffective as a lytic agent at high cell concentrations, and since it alone was used in cell lysate preparation, it was necessary to determine the optimum cell concentration for the assay (2.26). Table 4.3. shows the effect of swarm cell concentration on total assayable DNA polymerase activity, measured as the average (methyl-^{3}H)-thymidine incorporated into TCA-precipitable material per 250 µl assay volume. DNA polymerase activity was not proportional to the cell concentration, suggesting that lysozyme was limited in its effectiveness at higher cell concentrations. Consequently approximately 5 x 10^{8} cells were used in all further assays.

The effect of primer DNA concentration on DNA polymerase activity is shown in Fig. 4.8. At the lower concentrations of primer, a small increase in concentration had a large effect on incorporation, but above 60 µg ml^{-1} (incubation mixture), the system became saturated and further increases in primer incorporation did not stimulate incorporation.

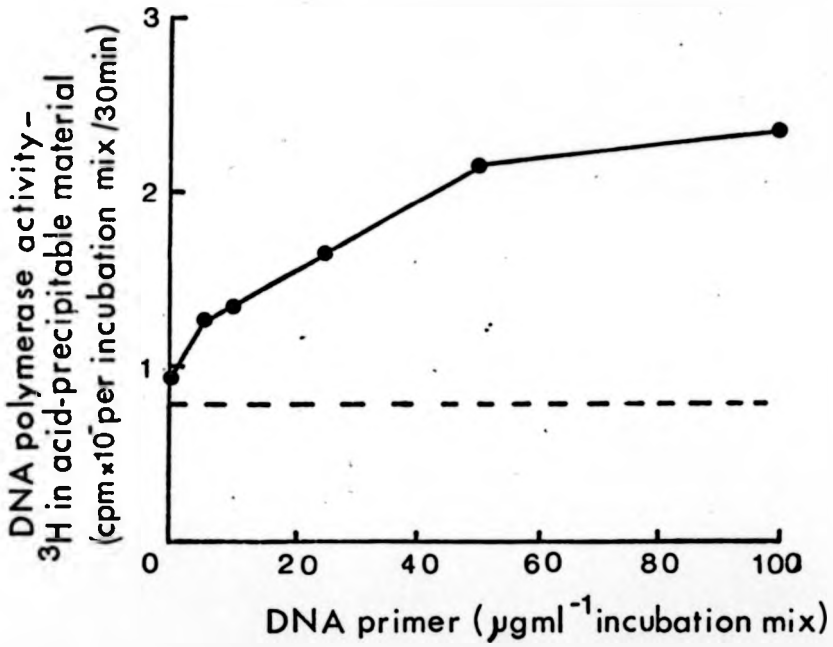


Fig. 4.8. The effect of primer DNA concentration on total DNA polymerase activity in the swarm cell

A swarm cell lysate (from ∿ 10^8 cells) was prepared (2.26), and incubated in the DNA polymerase assay mixture with varying amounts of 'activated' calf thymus primer DNA. The primer was activated by incubation with 0.1 μg/ml deoxyribonuclease I for 30 min at 37°C, and the reaction stopped by heating at 70°C.

The assay mixture was incubated at 37°C for 30 min, and incorporation was stopped by the addition of 10% TCA containing 0.1 M sodium pyrophosphate. T.C.A.-precipitable material was filtered onto GF/C filters, which were then washed, dried and counted.

Table 4.3. Effect of R.vannielii swarm cell concentration on total* DNA polymerase activity

Swarm cell number per ml.	[^{3}H]in acid-precipitable material (cp 10 min)
2×10^7	866
4×10^7	1510
4.3×10^8	3349
8.6×10^8	4060
2×10^9	1933
4×10^9	2064

The DNA polymerase activity in a 250 µl cell lysate was assayed as described in 2.26., and was measured as the radioactivity incorporated into T.C.A.-precipitable material at the end of the incubation period. A constant amount of primer DNA (0.5 µg) was used.

* total - the total activity attributable to all DNA polymerase enzymes in the cell

4.4.3. DNA polymerase activity during swarm cell morphogenesis and differentiation. DNA polymerase activity during swarm cell differentiation was measured as described in 2.26 ., and the results are shown in Fig. 4.9. Total polymerase activity was low during swarm cell maturation, but on stalk development a large increase in activity was observed. This could be correlated with the onset of DNA synthesis (4.2).

Table 4.4. shows the effect of sulfhydryl blocking agents on the radioactivity incorporated into TCA-precipitable material. Use of these agents allows the individual DNA polymerase activities to be distinguished in E.coli (Kornberg and Gefter, 1972), and by analogy should do the same in R.vannielii. The activities of both DNA polymerase I and III increased during swarm cell morphogenesis and differentiation. DNA polymerase II activity also appeared to increase. The results obtained are not reliable, since the experiment was not repeated, and the method (from E.coli) was used directly without modification. The application of this general method to R.vannielii must be fully investigated before any meaningful data are obtained.

4.5. Conclusions

The results presented in this chapter have provided information concerning DNA replication during swarm cell morphogenesis and differentiation in R.vannielii. ^{3}H-adenosine proved to be the most convenient isotope with which to monitor DNA replication.

Several important results have emerged from these studies. The first was that the time of initiation of DNA replication could be pinpointed to a period of approximately 20 minutes within a cell cycle of 6 hours (Fig. 4.3). Initiation was concomitant with the completion of stalk development, thus correlating an important biochemical event with a visible morphological 'landmark' event in the cell cycle. Having located the time of initiation of replication it should now be

Fig. 4.9. Total DNA polymerase activity during swarm cell morphogenesis and differentiation

○ growth (A_{540nm})

● total DNA polymerase activity/100 µl mix/30 min

Homogeneous swarm cell populations were selected (2.12) and incubated phototrophically at 30°C. At intervals 10 ml samples were withdrawn, centrifuged, 1 ml cell lysates prepared, and the total DNA polymerase activity in each extract (100 µl) was assayed (2.26). Growth rate and morphology of the culture were also examined.

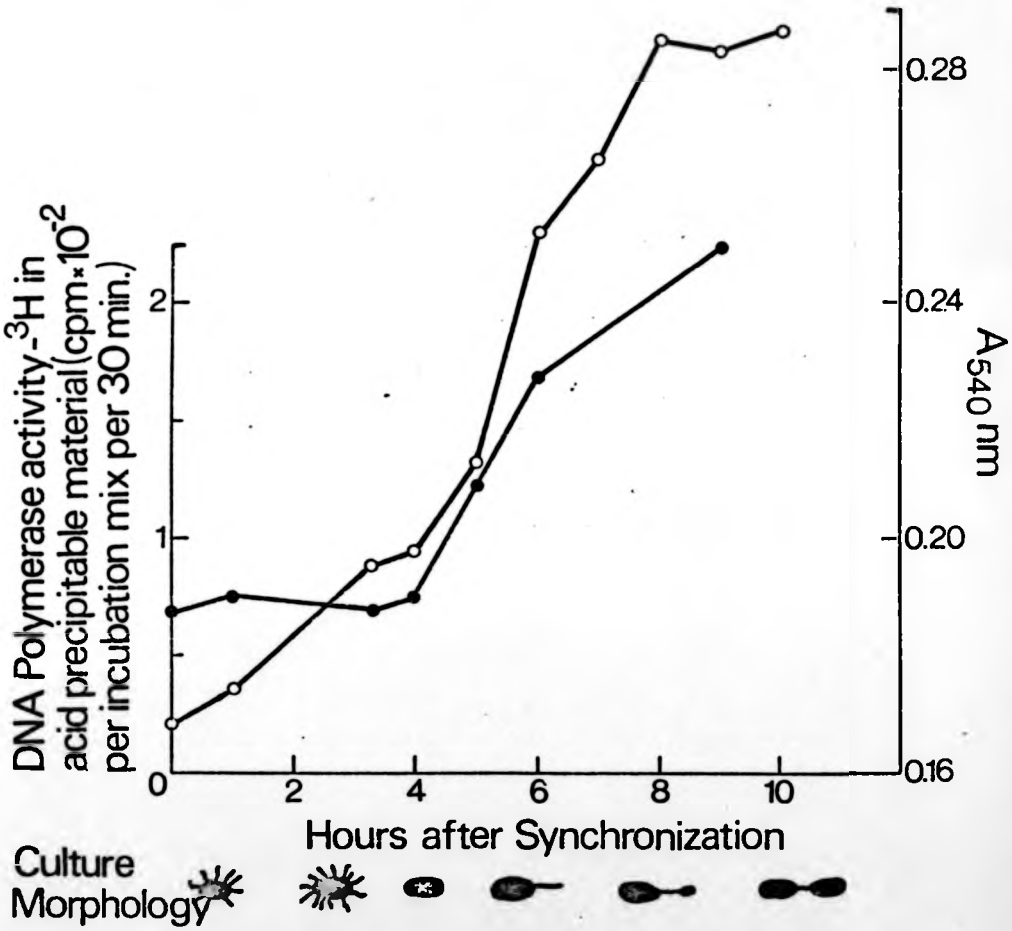

DNA Polymerase activity-^{3}H in acid precipitable material(cpm×10^{-2} per incubation mix per 30 min.)
0
1
2
0.16
0.20
0.24
0.28
A_{540} nm
0
2
4
6
8
10
Hours after Synchronization
Culture Morphology

Table 4.4. Activities of DNA polymerases I, II and III during swarm cell morphogenesis and differentiation

Time (hours after synchronization)	^3H in acid precipitable material (cp 10 min) total reaction mix	total +0.4 mM pCMS	total +2 mM NEM	radioactivity due to pol I	radioactivity due to pol II	radioactivity due to pol III
0	698	617	625	617	8	73
1	737	515	525	515	10	212
3.25	683	547	630	547	83	53
4	735	825	675	825	-	60
5	1223	1045	1150	1045	105	73
6	1695	1315	1495	1315	180	200
7	1860	1552	1520	1552	-	340
8	1430	1765	2247	1765	482	-
9	2225	2167	2002	2167	-	223
10	1752	1339	2042	1339	703	-

DNA polymerase activity in differentiating swarm cells (3×10^7) was assayed as described in 2.26. Assays were done in triplicate: 1) a total assay; 2) + 0.4 m M pCMS, which inhibits the activities of DNA polymerases II and III; 3) + 2 m M NEM which inhibits the activity of DNA polymerase III. Thus individual activities may be distinguished from the total as follows:- pol I activity = activity in the presence of pCMS; pol II activity = total - (pol I and pol III activities); pol III activity = total - activity in the presence of NEM.

This experiment was done once only, and needs modification before it provides meaningful results.

possible to preferentially label the origin of replication, leading subsequently to its isolation and characterization (7.4). DNA synthesis appeared to be continuous after the first division, but since division rapidly became asynchronous, this cannot be substantiated.

The second important point that emerged from the above study was that there was no DNA synthesis in the swarm cell. There was a period of swarm cell maturation (Fig. 4.3) during which no DNA replication occurred, and when reorganization essential for further development took place. DNA synthesis was repressed in the swarm cell, perhaps by the low levels of DNA polymerase enzymes, or due to the configuration of DNA.

Thirdly, DNA polymerase levels of activity were low in the swarm cell (Fig. 4.8). This could be due to either repression of the enzyme activity, for example by steric hindrance, or the presence of a small amount of enzyme, giving rise to the need for further de novo synthesis for increased activity. These possibilities cannot be distinguished without measuring the absolute amount of enzyme after purification, or without a knowledge of the specific mRNA molecules for DNA polymerases.

The level of DNA polymerase activity is likely to be important in the repression of DNA synthesis in the swarm cell. It may form part of the control mechanism which ensures that DNA replication does not occur until the stalked cell is about to bud. In B.subtilis, DNA polymerase III activity increases when spores are activated to germinate, which led to the speculation that the enzyme is involved in the control of DNA replication (Ciarrochi et al., 1977). Further work in this area is needed.

The fourth main point to be taken from these results is that RNA synthesis did occur in swarm cells which were inhibited in their differentiation by the lack of light. The inhibitory effect was

powerful, and ensured that 90% of the swarm cells remained in the motile phase. Nonetheless, once morphogenesis and differentiation were initiated, the swarm cell was able to progress through the whole sequence, indicating that the light-dependent step(s) were at the transition from the motile swarm cell to the stationary cell (Potts and Dow, 1979).

Since metabolic activity in the inhibited swarm cell was low, it may be assumed that little rRNA synthesis was occurring. Swarm cell-specific soluble proteins have been detected by ^{35}S-methionine pulse labelling and polyacrylamide gel electrophoresis (France and Dow, unpublished data; Fig. 1.19), indicating that several high molecular weight proteins disappear as differentiation proceeds. One may suggest that the RNA synthesized during swarm cell inhibition may contain a high proportion of mRNA for swarm cell-specific proteins, and that synthesis of these proteins is required for subsequent swarm cell differentiation. It would be interesting to determine whether these proteins are still made in the swarm cell after prolonged incubation in the dark. RNA specific to either cell type, and RNA common to both, should be detectable by separate hybridization of swarm cell RNA and mother cell RNA to swarm cell DNA.

Although R.vannielii is similar to C.crescentus (Degnen and Newton, 1972b) and R.palustris (Westmacott and Primrose, 1977) in that DNA synthesis is a characteristic of the stalked cell only, it has more potential than either of these as a model for differentiation. R.vannielii shows a clearly defined differentiation sequence which can be completely inhibited by the absence of light, and so can be easily manipulated experimentally. Initial biochemical studies have revealed complex mechanisms of regulation, which may now be investigated more fully by modern biological techniques.

CHAPTER 5

The effect of metabolic inhibitors on swarm cell morphogenesis and differentiation

5.1. Introduction

Studies of the kinetics of nucleic acid synthesis during swarm cell morphogenesis and differentiation revealed interesting facts about the role of the swarm cell in these processes. Moreover, they indicated that a complex set of regulatory mechanisms operates during the swarm cell differentiation sequence. This chapter describes work in which the effect of specific metabolic inhibitors on the ensuing morphogenesis and differentiation was examined.

Metabolic inhibitors have been used by other workers in the elucidation of the regulation of cell cycle events in *E.coli* (Ward and Glaser, 1970; Doudney, 1978), *C.crescentus* (Degnen and Newton, 1972b), *Myxococcus* (Kimichi and Rosenberg, 1976) and *R.palustris* (Westmacott and Primrose, 1977). The rationale behind most of these experiments was to inhibit one specific function in the bacterium, such as DNA synthesis, and then to examine the effect of this on other measurable biochemical parameters, and on the morphological 'landmarks' in the cell cycle. This was the method used to establish that cell division was dependent upon the completion of chromosome replication in *C.crescentus* (Degnen and Newton, 1972b).

Many metabolic inhibitors are known and used in biochemical research, but the mode of action of some, particularly inhibitors of nucleic acid synthesis, is uncertain (Kersten and Kersten, 1974). In this study, the effects of two metabolic inhibitors, namely nalidixic acid (a DNA synthesis inhibitor) and chloramphenicol (a protein synthesis inhibitor), on swarm cell morphogenesis and differentiation, were examined.

5.2. Effect of nalidixic acid on swarm cell morphogenesis and differentiation

5.2.1. Introduction. Nalidixic acid was chosen to examine the relationship between DNA replication and cell division in R.vannielii. The drug is an inhibitor of DNA synthesis (Goss et al., 1965) but at high concentrations it may also affect RNA synthesis (Javor, 1974). Recent workers detected a mutation in the nal A gene, which results in a mutant DNA gyrase enzyme. This enzyme is responsible for the supercoiling of DNA in E.coli, and acts by introducing negative superhelical turns in an ATP-dependent reaction (Gellert et al., 1976). The fact that the nal A gene is involved in the production of this enzyme suggests that nalidixic acid acts by inhibiting the nicking-closing activity of the enzyme (Sugino et al., 1977; Gellert et al., 1977).

5.2.2. Effect of nalidixic acid on swarm cell growth and morphology. Homogeneous populations of swarm cells were obtained by selection (2.12), incubated for 16 hours with varying concentrations of nalidixic acid, and then examined by phase contrast microscopy and electron microscopy. The cell volume distribution of each culture was determined using a Coulter counter and channelyser (2.14).

The effect of nalidixic acid on the morphology of the developing swarm cell is shown in Figs. 5.1 and 5.2. Below 20 μg ml^{-1}, nalidixic acid had little effect on morphology, but at higher concentrations an increasing proportion of the cells became elongated and distorted in shape. There was no cell division, as indicated by a lack of plug formation in these distorted cells. The proportion of cells which appeared elongated increased with the concentration of nalidixic acid (Table 5.1). Daughter cells formed but these also became elongated.

Fig. 5.1. Light micrographs of nalidixic acid-treated swarm cells.

Homogeneous swarm cells were incubated phototrophically at 30°C with varying concentrations of nalidixic acid for 16 hours, and then examined by phase contrast microscopy.

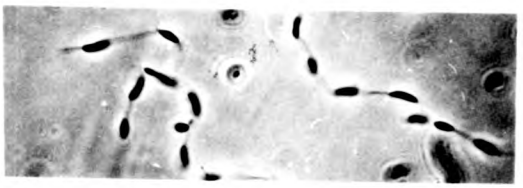
Magnification = 6000 x .

NaI($\mu g ml^{-1}$)

0

50

75



100

150

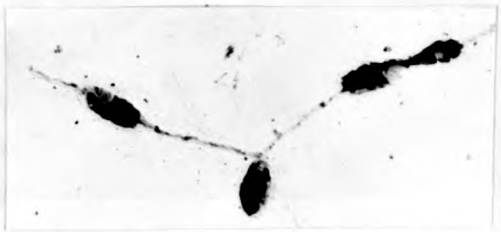
Fig. 5.2. Electron micrographs of nalidixic acid-treated swarm cells.

Homogeneous swarm cells were incubated phototrophically at 30°C with varying concentrations of nalidixic acid for 16 hours. Cell samples were shadowed with gold-palladium, and examined in the electron microscope.

Magnification = 8000 x.

NaI ($\mu g ml^{-1}$)

0



50

75

100

150

Table 5.1. Effect of nalidixic acid concentration on the proportion of elongated R.vannielii cells at 16 hr. 'end point analysis'

Nalidixic acid concentration	% elongated cells in culture after 15 hrs.
0	0
10	15
30	46
50	60
70	75
90	80
100	92

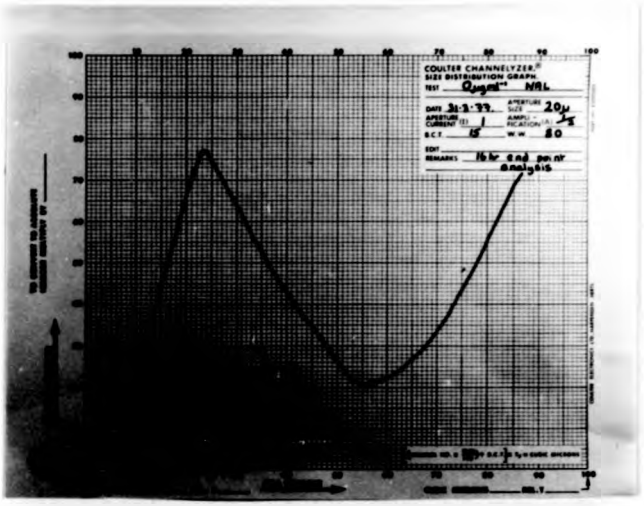
Homogeneous swarm cells were selected by filtration (2.12), and incubated phototrophically at 30°C with varying concentrations of nalidixic acid for 16 hours. The cultures were then examined by phase contrast microscopy. Percentages recorded are an average of three estimations.

At concentrations greater than 150 μg ml^{-1}, swarm cell differentiation was arrested at the stage of stalk synthesis, indicating that at these concentrations nalidixic acid affects other metabolic processes as well as those responsible for cell growth and elongation. The electron micrographs (Fig. 5.2) show that plug formation was inhibited by nalidixic acid, and so there was no physiological separation of mother and daughter cell.

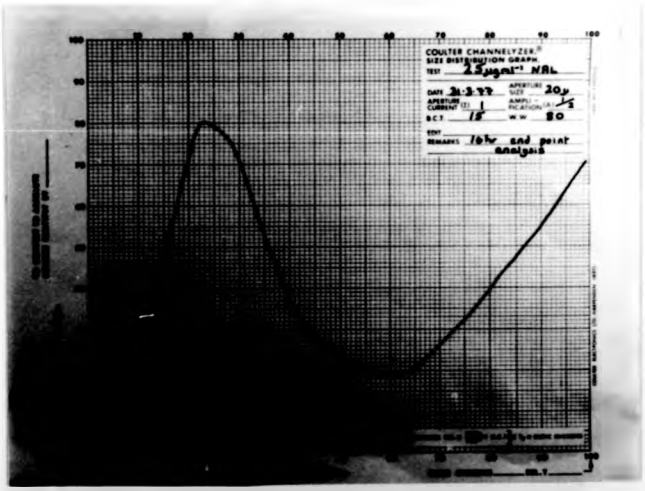
An analysis of the cell volume distribution confirmed that the major effect of nalidixic acid was to cause gross morphological distortion and cell enlargement. Fig. 5.3 shows the cell volume distribution profiles of nalidixic acid-treated cells compared with control cells, indicating a progressive shift in average cell volume with increasing concentrations of the drug.

The effect of nalidixic acid on the growth of homogeneous swarm cell populations in a cuvette culture system (2.11) is shown in Fig. 5.4. Growth of the control culture was stepwise for 20 hours, and then became exponential. This was because the second and third rounds of division retained some degree of synchrony from the first, but as the number of divisions increased, the culture became completely asynchronous, and exponential growth ensued.

The absolute effect of nalidixic acid on the growth rate was not very marked, but the growth characteristics of the swarm cell were altered. At 50 μg ml^{-1} nalidixic acid, there was some inhibition of growth, while at 100 μg ml^{-1} there was a marked inhibition after 20 hours. A plateau was observed during the mid exponential phase of growth, unlike the stepwise increase seen in the control population. These results indicated that protein synthesis continued in nalidixic acid-treated cells, but some processes were affected which



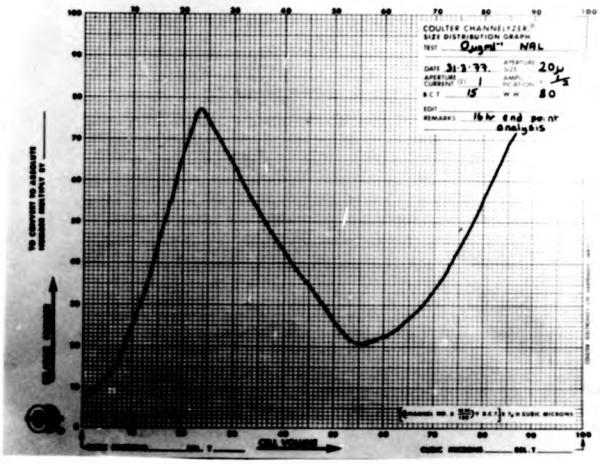
a) 0 µg ml^{-1} NAL



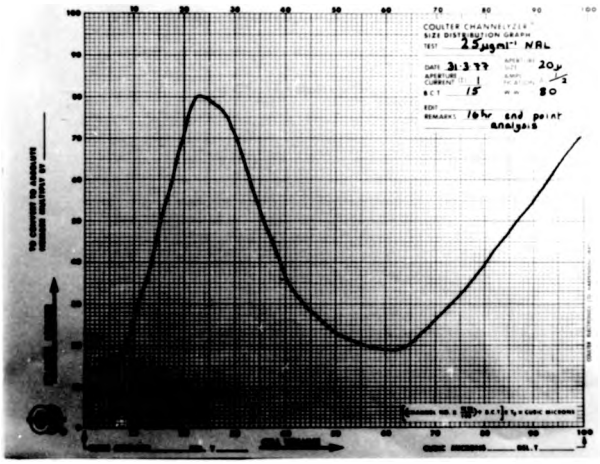
b) 25 µg ml^{-1} NAL

Fig. 5.3. Cell volume distribution of swarm cells incubated with nalidixic acid.

Aliquots from swarm cell populations which had been incubated phototrophically at 30°C with varying concentrations of nalidixic acid for 16 hours were diluted and their cell volume distribution was determined in the Coulter counter and channelyzer (2.14).



a) 0 μg ml^{-1} NAL

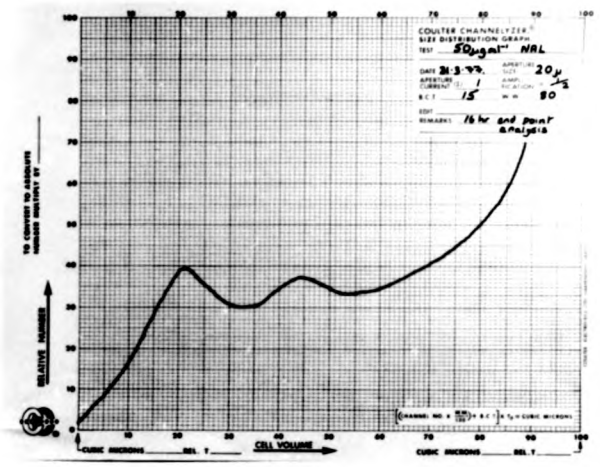


b) 25 μg ml^{-1} NAL

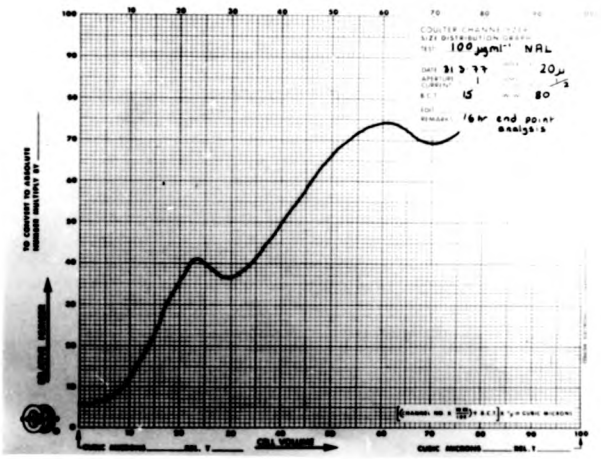
Fig. 5.3. Cell volume distribution of swarm cells incubated with nalidixic acid.

Aliquots from swarm cell populations which had been incubated phototrophically at 30°C with varying concentrations of nalidixic acid for 16 hours were diluted and their cell volume distribution was determined in the Coulter counter and channelyzer (2.14).

Fig. 5.3. (continued)



c) 50 µg ml^{-1} NAL



d) 100 µg ml^{-1} NAL

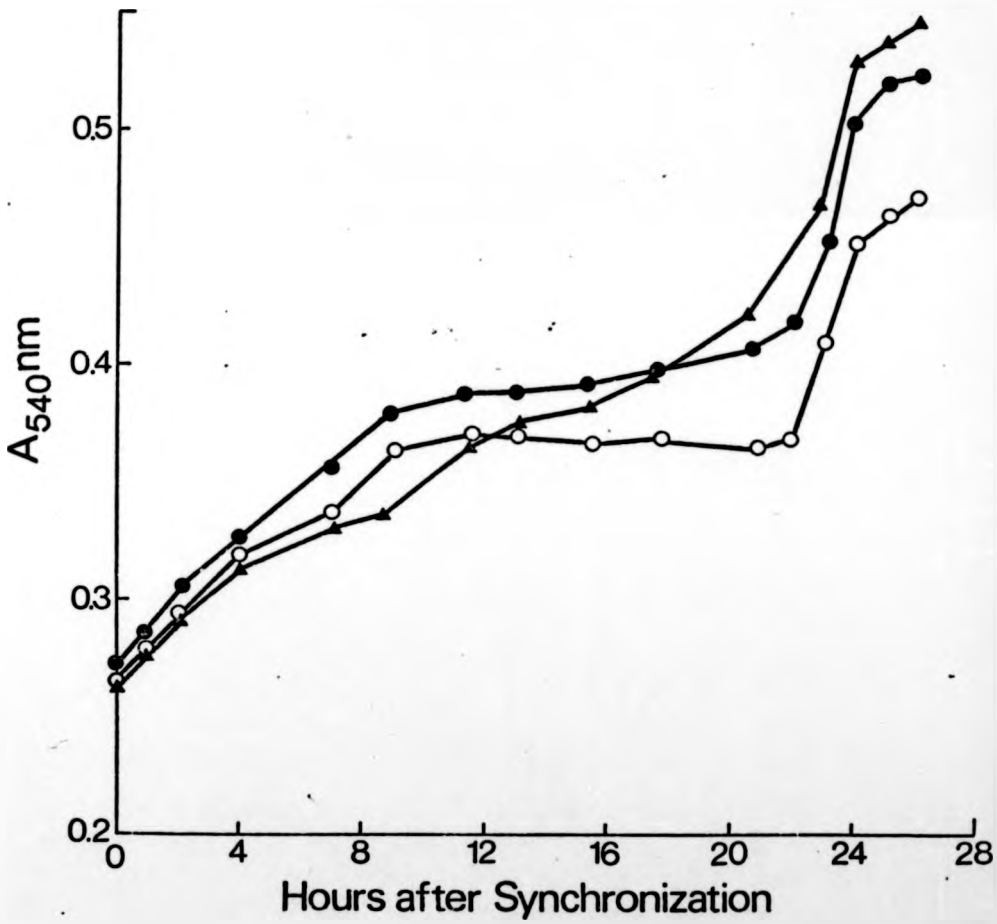
Fig. 5.4. Effect of nalidixic acid on the growth of homogeneous swarm cells of R.vannielii.

▲ 0 µg ml^{-1} nalidixic acid

● 50 " " "

o 100 " " "

Homogeneous populations of swarm cells (1.5 x 10^7/ml) were selected by filtration (2.12), and incubated phototrophically at 30°C with 0, 50 or 100 µg ml^{-1} nalidixic acid, in 1 cm cuvettes. The A_{540nm} of each culture was measured at intervals for 28 hours.


0.5
0.4
0.3
0.2
A_{540}nm
0
4
8
12
16
20
24
28
Hours after Synchronization

altered the metabolic behaviour of the cells, and led to cell distortion and an abnormal growth pattern.

5.2.3. Effect of nalidixic acid on nucleic acid and protein synthesis during swarm cell morphogenesis and differentiation. Homogeneous swarm cell populations were incubated with 0, 50 or 100 μg ml^{-1} nalidixic acid, and nucleic acid synthesis was measured by following cellular incorporation of exogenous ^{3}H-adenosine (2.27). Protein synthesis was followed by measuring the cellular incorporation of ^{35}S-methionine (2.27.3). The results are presented in Fig. 5.5. In control cultures, incorporation of radiolabel into nucleic acids followed the same pattern as observed earlier (4.3.2), but the onset of DNA synthesis occurred after 4 hours, rather than after 2 hours as found previously. This was probably a result of the difference in physiological state of the cells at the time of selection. Although swarm cells are selected at approximately the same time in each experiment, some populations may be younger than others in terms of membrane biogenesis, for example, and so a longer period of maturation may elapse, after initiation of differentiation, before DNA synthesis commences.

At 50 μg ml^{-1}, nalidixic acid inhibited DNA replication, whilst having no effect on protein and RNA synthesis, but at 100 μg ml^{-1} the drug inhibited DNA, RNA and protein synthesis. Both RNA and protein synthesis were inhibited only after 3-4 hours' incubation, since initially incorporation of the radiolabelled precursors was the same as in the control culture.

5.2.4. Discussion. The treatment of homogeneous swarm cell populations of R.vannielii with 50 μg ml^{-1} nalidixic acid had three marked effects :-

Fig. 5.5. Effect of nalidixic acid on nucleic acid and protein synthesis during swarm cell morphogenesis and differentiation.

▲ 0 μg ml^{-1} nalidixic acid

● 50 " " "

o 100 " " "

A homogeneous population of swarm cells was selected by filtration (2.12), and incubated phototrophically at 30°C with 0, 50 or 100 μg ml^{-1} nalidixic acid. Nucleic acids in the cultures were radiolabelled by incubation with 5 μCi ml^{-1} ^{3}H-adenosine in 5 μM adenosine carrier, and proteins by 0.1 μCi ml^{-1} ^{35}S-methionine in 0.0 3mM methionine. At 30 min. intervals, 15 ml of each culture was withdrawn and split into two equal amounts. Incorporation in both was stopped by the addition of 5% TCA. One sample was hydrolysed in 1 M NaOH overnight at 37°C, and TCA-precipitable material remaining was collected on GF/C filters for an estimate of incorporation into DNA. TCA-precipitable material in the other sample was collected on GF/C filters, for an estimate of incorporation into protein and RNA (2.27). Counts attributable to DNA were subtracted from the total acid-precipitable [^{3}H] count, to give those counts due to RNA.

Standard amounts of [^{3}H] and [^{35}S] were counted before and after mixing, to estimate the amount of 'spill-over' into each channel from each isotope. All counts were adjusted accordingly.

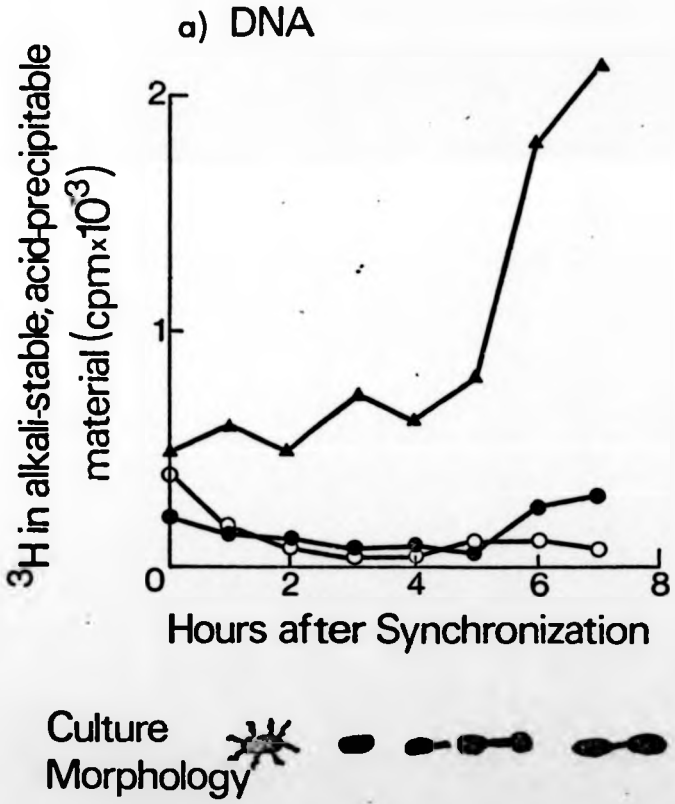
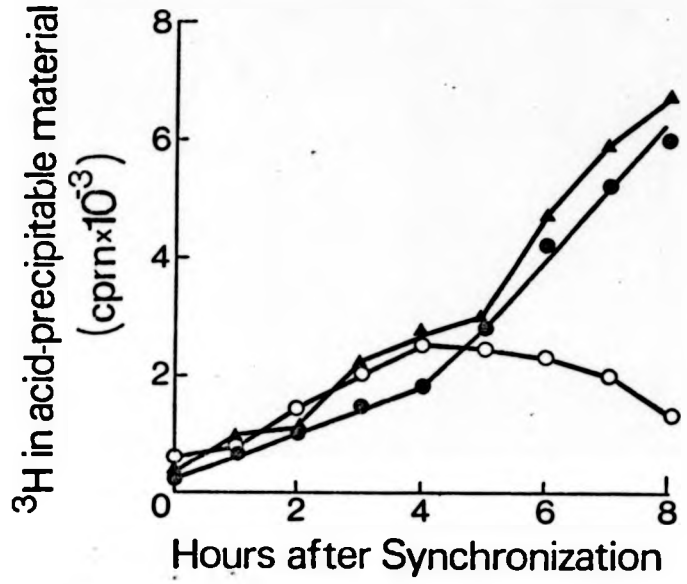
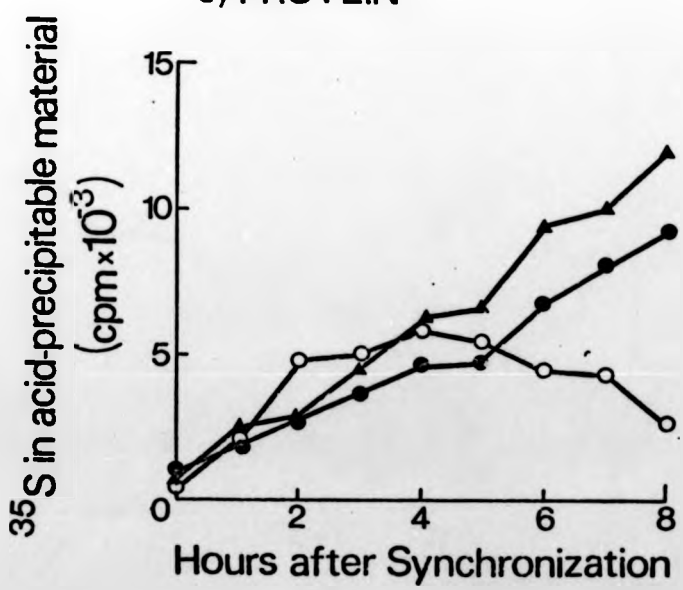

a) DNA
^{3}H in alkali-stable, acid-precipitable material (cpm×10^3)
2
1
0
2
4
6
8
Hours after Synchronization
Culture Morphology

Fig. 5.5. (continued)

b) RNA



c) PROTEIN



a) DNA replication was completely inhibited,

b) cell division was inhibited,

and c) cell growth continued, but was distorted.

At higher concentrations (~100 μg ml^{-1}) the antibiotic also caused some inhibition of RNA and protein synthesis, but the development of grossly distorted cells was still possible (Fig. 5.2). Since nalidixic acid inhibits both DNA and RNA synthesis, the DNA gyrase enzyme, which it has been shown to affect, may have a role in transcription, as well as in DNA replication.

Although 50 μg ml^{-1} nalidixic acid inhibited DNA synthesis, daughter cells were produced, indicating that the initiation of daughter cell formation was under the control of the mother cell genome. DNA replication was required for cell division, since cross walls were not formed between mother and daughter cells after nalidixic acid treatment. Cross wall formation was therefore under the direct control of the daughter cell genome.

This suggestion was made previously by Whittenbury and Dow (1977), based on the evidence that whatever the nutrient conditions, cross walls were always formed at the same distance from the daughter cell, the distance from cross wall to mother cell varying widely. The above evidence substantiates this suggestion.

The distortion seen in nalidixic acid-treated cells could be due to uncontrolled growth, and growth becomes increasingly haphazard. Thus one could postulate that growth is initiated by the mother cell genome, but is ultimately subject to control by the daughter cell genome. In the absence of a daughter cell genome, the daughter cell becomes distorted, due to uncontrolled protein synthesis.

The completion of chromosome replication is a necessary prerequisite for cell division in E.coli (Helmstetter and Pierucci, 1968), C.crescentus (Degnen and Newton, 1972b) and R.palustris (Westmacott and Primrose, 1977). In B.subtilis however, cell division may continue in the absence of protein synthesis, to produce anucleate cells (Donachie et al., 1971). This variation may reflect a fundamental difference between Gram-positive and Gram-negative bacteria, as it has been shown that R.vannielii follows the Gram-negative pattern.

These data show that in R.vannielii a 'dependent pathway' control mechanism is operating, in which chromosome replication is required for cell division. Since chromosome replication always follows stalk formation these events can be linked in a dependent pathway (Fig. 5.6). As yet, no information is available regarding the involvement of other processes, e.g. flagella assembly or membrane biogenesis, in this pathway or in other ancillary pathways.

Future experiments with nalidixic acid should examine the effect of the antibiotic at different stages in swarm cell morphogenesis and differentiation. Since the effects of nalidixic acid are reversible it would be possible to investigate the effect of removing the drug during the differentiation sequence. Other DNA synthesis inhibitors such as hydroxyurea and mitomycin C may help elucidate the regulation of differentiation.

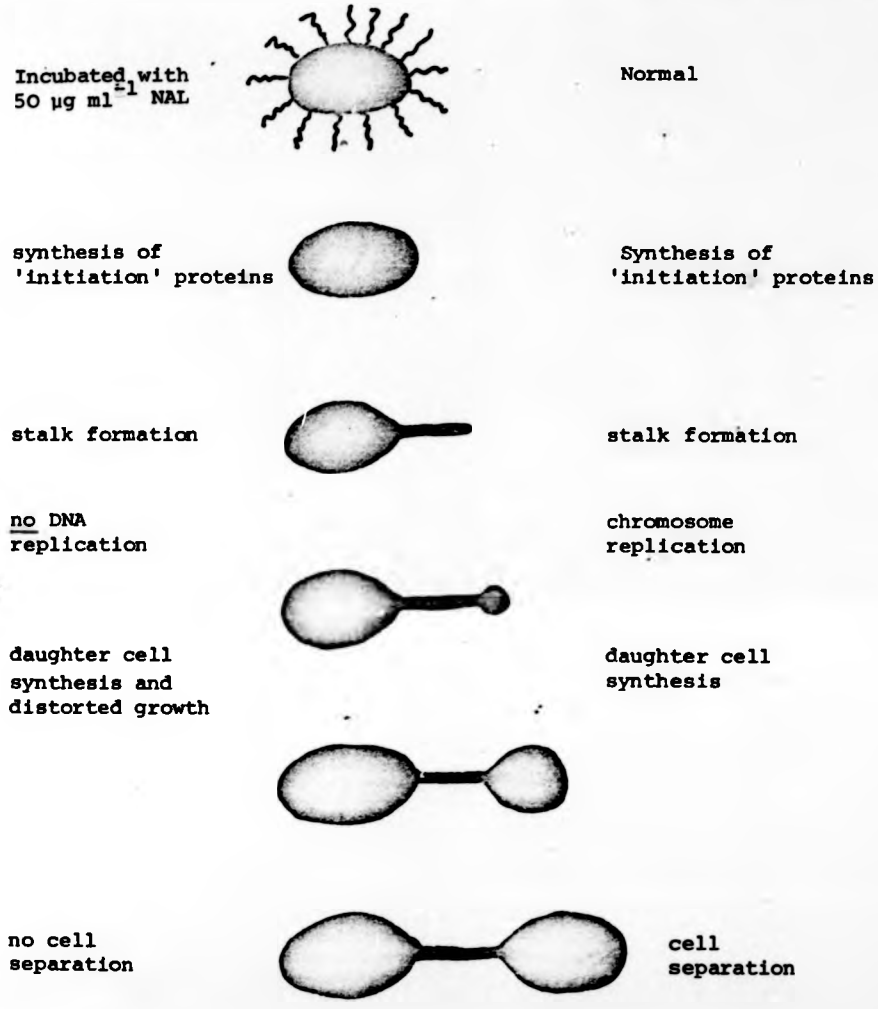


Fig. 5.6. Linkage of cell cycle events in a 'dependent pathway' in R.vannielii.

5.3. Effect of chloramphenicol on DNA replication in R.vannielii

5.3.1. Introduction. The observation (4.3.2) that there is a lag period in the swarm cell differentiation sequence, when no DNA replication occurs, suggests that essential requirements for replication are synthesized during this time. Protein synthesis may be essential during this period, and so the dependence of nucleic acid synthesis on protein synthesis was examined with the use of a protein synthesis inhibitor.

Many inhibitors of protein synthesis are known, but chloramphenicol was chosen for these experiments since a) it has a powerful inhibitory effect in prokaryotes, and b) its mode of action is well established. Chloramphenicol inhibits translation by interacting with the 50s subunit of the prokaryotic ribosome (Vasquez, 1964). It is ineffective in the eukaryotic cell nucleus.

5.3.2. Effect of chloramphenicol on growth rate, and on nucleic acid and protein synthesis, in an exponential 'simplified cell cycle' culture of R.vannielii. An exponentially growing culture was used in the initial experiments to establish that chloramphenicol had an effect on nucleic acid synthesis via its effect on protein synthesis, and also to allow an estimate of the DNA replication time (S period) in R.vannielii to be made.

It had previously been shown that 15 μg ml^{-1} chloramphenicol inhibits protein synthesis in R.vannielii immediately and completely (C. S. Dow, personal communication). The growth rate (as measured by the increase in A_{540nm} with time) of parallel 'simplified cell cycle' cultures of R.vannielii was measured, and protein and nucleic acid synthesis in both cultures was monitored by measurement of the cellular uptake of ^{35}S-methionine and ^{3}H-adenosine respectively. After 4 hours, chloramphenicol (15 μg ml^{-1}) was added to one culture,

and measurements were continued.

As indicated in Fig. 5.7, when chloramphenicol was added to one culture, growth of that culture was halted. Incorporation of ^{35}S-methionine into protein ceased (Fig. 5.8a), indicating that protein synthesis had been completely inhibited. In the control culture, both parameters continued to increase exponentially.

The effect of chloramphenicol on nucleic acid synthesis is shown in Fig. 5.8 (b) and (c), RNA synthesis continuing for 1-2 hours before inhibition was observed. Incorporation of ^{3}H-adenosine into DNA continued for 1 hour after the addition of the antibiotic, and then levelled off, indicating that DNA replication was able to continue for 1 hour in the presence of chloramphenicol, but then ceased.

This result suggested that lack of protein synthesis inhibited the initiation of DNA replication, but did not affect the ongoing rounds of replication. These were completed, allowing one to estimate that the length of DNA replication in R.vannielii was approximately 1 hour.

5.3.3. Effect of L-threo chloramphenicol on protein and DNA synthesis in an exponential 'simplified cell cycle' culture. The indirect effect of chloramphenicol on DNA synthesis demonstrated above could possibly be due, not to the inhibition of protein synthesis, but to some other inhibitory effect chloramphenicol might have on membrane function. This has been demonstrated in plants, where the photosynthetic apparatus was affected, and may also be the case in photosynthetic bacteria.

One could explain the results in 5.3.2. by postulating that the observed inhibition of DNA replication was due to the alteration of

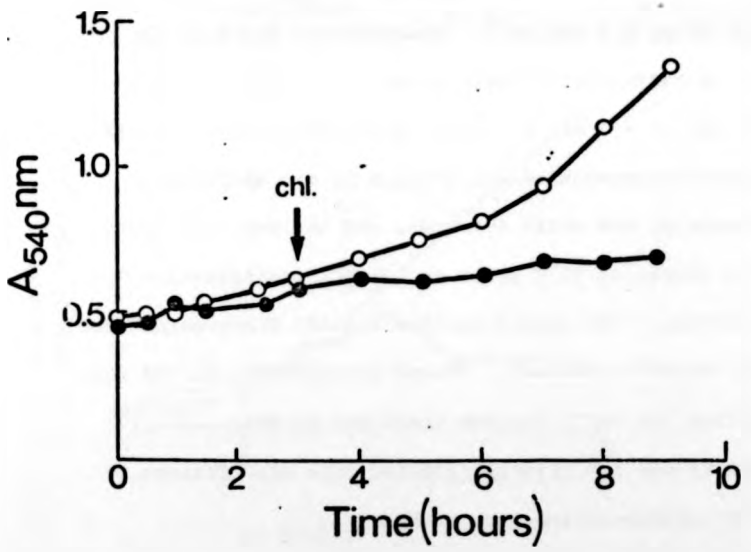


Fig. 5.7. Effect of chloramphenicol on the growth rate of a 'simplified cell cycle' culture of R.vannielii

● 15 μg ml^{-1} chloramphenicol added at hour 4

o no chloramphenicol added

Two parallel early-exponential cultures (A_{540nm}=0.5) were incubated phototrophically at 30°C. At regular intervals the A_{540nm} of each culture was measured. After 4 hours incubation, 15 μg ml^{-1} chloramphenicol was added to one of the cultures, and the measurement of A_{540nm} from both was continued.

Fig. 5.8. Effect of chloramphenicol on nucleic acid and protein synthesis in a 'simplified cell cycle' culture of R.vannielii

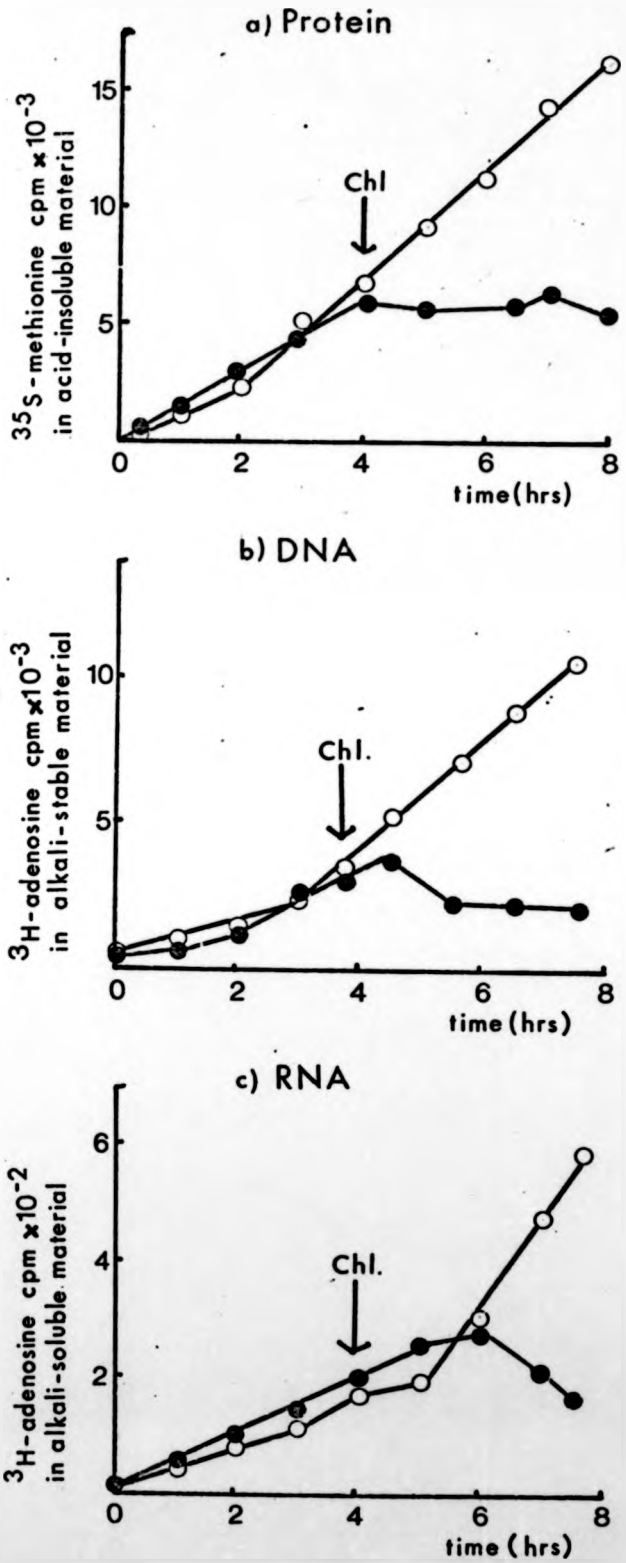
● 15 μg ml^{-1} chloramphenicol added at hour 4

o no chloramphenicol added

Four parallel early-exponential cultures (A_{540nm}=0.5) were incubated phototrophically at 30°C, two in the presence of a) 5 μCi ml^{-1} ^{3}H-adenosine and 5 μ M adenosine, and two in the presence of b) 0.1 μCi ml^{-1} ^{35}S-methionine and 0.03 mM methionine, to radiolabel nucleic acids and proteins, respectively. Samples (a - 2 ml, b - 1 ml) were withdrawn at regular intervals, and incorporation was stopped by the addition of 5% TCA. Sample a) was split into two, and one was hydrolysed in M NaOH overnight at 37°C prior to 10% TCA-precipitation onto GF/C filters. The second portion was 10% TCA-precipitated immediately onto GF/C filters. Counts attributable to DNA were subtracted from the total to give those due to RNA.

Sample b) was 10% TCA-precipitated onto GF/C filters to measure ^{35}S -incorporation into protein.

After 4 hours incubation, 15 μg ml^{-1} chloramphenicol was added to two cultures, one from a) and one from b), and sampling at intervals was resumed.


a) Protein
^{35}S-methionine cpm ×10^{-3} in acid-insoluble material
Chl
time(hrs)
b) DNA
^{3}H-adenosine cpm ×10^{-3} in alkali-stable material
Chl.
time (hrs)
c) RNA
^{3}H-adenosine cpm ×10^{-2} in alkali-soluble material
Chl.
time (hrs)

membrane systems which perhaps changed or prevented the DNA-membrane attachment which is necessary for replication. This possibility was examined by the use of L-threo chloramphenicol. Inhibition of protein synthesis is stereo-specific, and only the D-threo isomer is active (Vasquez, 1979). Therefore, the L-threo isomer, although affecting membranes in the same way as the D-isomer, would not affect protein synthesis.

The experiment described in 5.3.2. was repeated with L-threo chloramphenicol and the results are shown in Fig. 5.9. Only protein and DNA synthesis were monitored. Incorporation of ^{35}S-methionine and ^{3}H-adenosine into protein and DNA respectively was similar in the control culture and the L-threo chloramphenicol-treated culture. There was little difference in the final A_{540nm} of both cultures. Consequently the effects on DNA replication described in 5.3.2. can be attributed to the inhibition of protein synthesis by chloramphenicol, rather than to any effect on membrane transport.

5.3.4. Effect of chloramphenicol on DNA replication during swarm cell morphogenesis and differentiation. The idea that ongoing rounds of replication are unaffected by chloramphenicol was tested by adding the antibiotic at different times during the DNA replication cycle. This was done by adding chloramphenicol at specific times during the differentiation sequence of homogeneous swarm cell populations, since the time of initiation of DNA synthesis has been located (4.5).

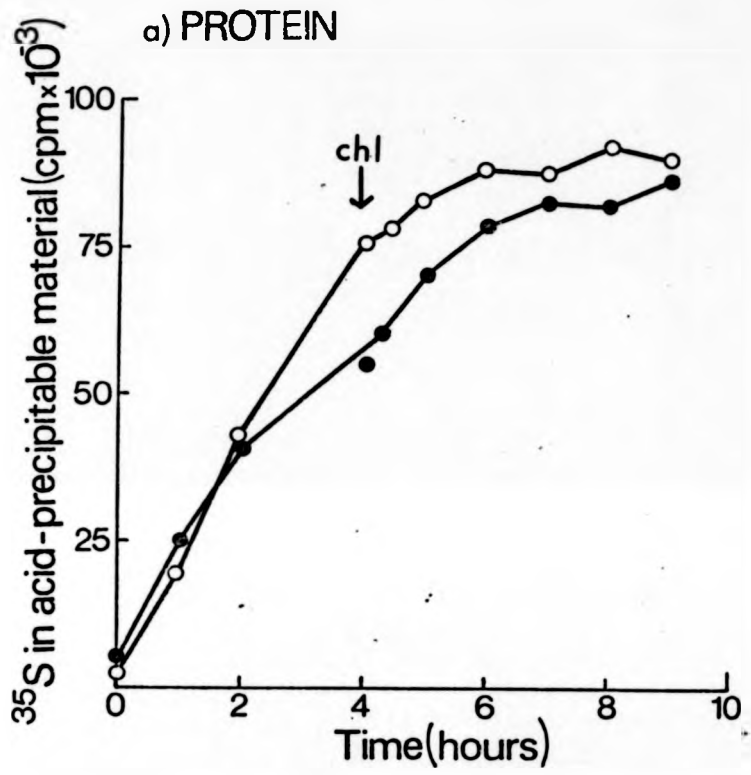
The effect of chloramphenicol addition immediately before initiation of replication, and during elongation of the DNA strands, was investigated, the experimental procedure being essentially the

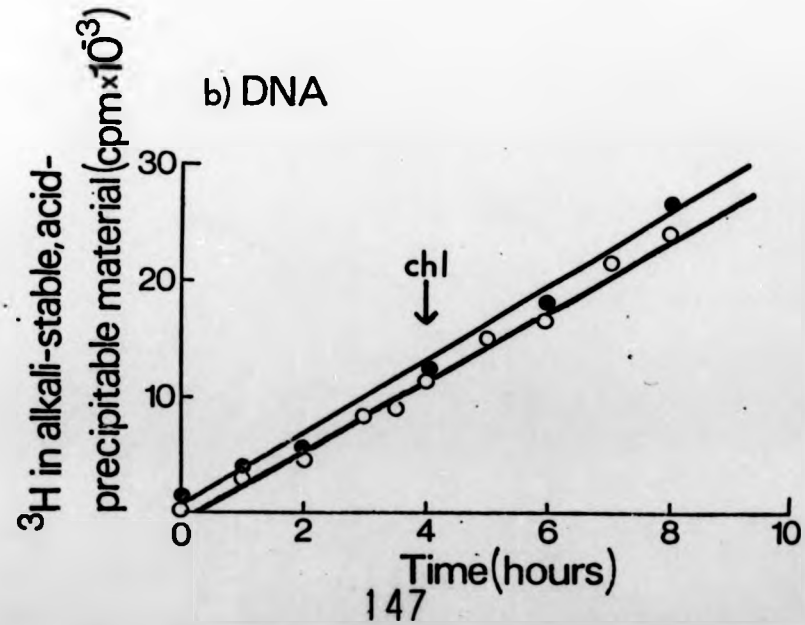
Fig. 5.9. Effect of L-threo chloramphenicol on protein and DNA synthesis in the 'simplified cell cycle' expression of R.vannielii

● 15 μg ml^{-1} L-threo chloramphenicol added at hour 4

o no chloramphenicol added

The experimental procedure in this experiment was the same as in Fig. 5.8., except that the culture used was in exponential phase (A_{540nm} ~2.0), and 0.4 μCi ml^{-1} ^{35}S-methionine was used to radiolabel proteins. RNA synthesis was not measured. After 4 hours, 15 μg ml^{-1} L-threo chloramphenicol was added to two of the cultures, and sampling at intervals from all cultures was resumed.


a) PROTEIN
^{35}S in acid-precipitable material (cpm×10^{-3})
100
75
50
25
0
2
4
6
8
10
Time (hours)
chl


b) DNA
^{3}H in alkali-stable, acid-precipitable material (cpm×10^{-3})
30
20
10
0
2
4
6
8
10
Time (hours)
chl

same as for the exponential culture, except that a larger sample size was used. Protein synthesis in the swarm cell was completely inhibited by 15 μg ml^{-1} chloramphenicol.

Fig. 5.10. shows that when the antibiotic was added 1½ hr. after selection, that is, before the initiation of replication, no DNA synthesis took place. However, when chloramphenicol was added later, at hour 3 or 4, DNA synthesis continued for about 1 hour before stopping.

Thus, once DNA replication had been initiated, it continued until the end of that round of synthesis in the presence of chloramphenicol. No further rounds of replication could be initiated, and consequently incorporation levelled off. These results show that protein synthesis was required for the initiation but not for the continuation of DNA replication in R.vannielii.

5.3.5. Discussion. Use of the specific protein synthesis inhibitor chloramphenicol has demonstrated that chromosome replication in R.vannielii has an obligate requirement for protein synthesis. Once initiation has occurred, completion of DNA replication proceeds in the absence of protein synthesis.

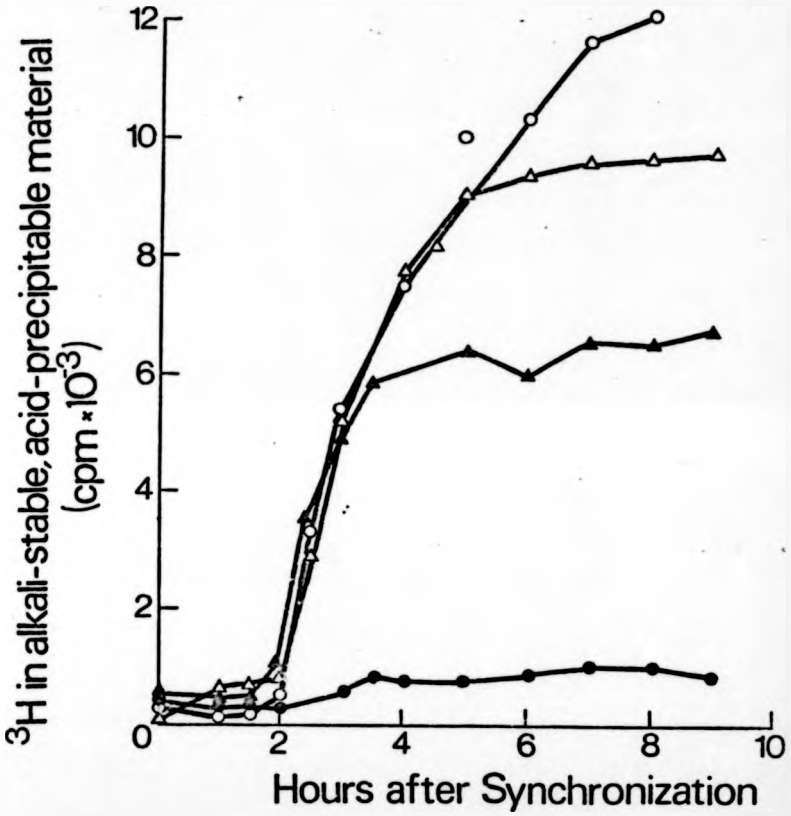
It was possible, from the experiments in 5.3.2, to make an estimate of the duration of the DNA replication time, i.e. the S phase of the cell cycle. Since the round of replication just initiated when chloramphenicol was added, finished last, the S period was estimated to be approximately 60 min. This was a longer time than the corresponding period in E.coli (40 min.) as determined by the Helmstetter technique (Cooper and Helmstetter, 1968), but this was to be expected since the duration of the cell cycle in R.vannielii is much longer than that in E.coli.

Fig. 5.10. Effect of chloramphenicol on DNA replication during swarm cell morphogenesis and differentiation.

- o no chloramphenicol added
- ● 15 µg ml^{-1} chloramphenicol added at 1.5 hours
- ▲ " " " " 3 hours
- △ " " " " 4 hours

A homogeneous population of swarm cells was selected by filtration (2.12) and split into 4 smaller cultures. Each was incubated phototrophically at 30°C in the presence of 5 µCi ml^{-1} ^{3}H-adenosine and 5 µM adenosine. At regular intervals 5 ml samples were withdrawn, and incorporation was stopped by the addition of 5% TCA. After hydrolysis in 1 M NaOH overnight at 37°C, 10% TCA-precipitable material remaining was collected on GF/C filters. These were washed, dried and counted.

15 µg ml^{-1} chloramphenicol was added to three of the cultures at the times indicated above, and sampling was continued.


^{3}H in alkali-stable, acid-precipitable material (cpm * 10^{-3})
12
10
8
6
4
2
0
2
4
6
8
10
Hours after Synchronization

The requirement for protein synthesis in the initiation of replication during swarm cell morphogenesis and differentiation has been located temporally between the loss of flagellae and the completion of stalk development (Fig. 5.11). It should be possible to detect specific 'initiation proteins' by polyacrylamide gel electrophoresis and autoradiography of proteins from different stages of swarm cell differentiation. Although preliminary work has shown detectable differences in protein patterns (Fig. 1.19; Dow and France, unpublished observations), no 'initiation proteins' have yet been identified.

In C.crescentus, Olsey and Newton (1978) have identified a protein synthesis requirement for the initiation of DNA synthesis, and have also located a separate temperature-sensitive gene product requirement. They find that both of these are necessary for DNA replication in both cellular expressions of the bacterium. Protein synthesis is required for DNA replication in other bacteria (Lark, 1969), and in yeast (Hereford and Hartwell, 1973).

It has been suggested that protein synthesis is required for the termination of replication (Marunouchi and Messer, 1973), although later data do not substantiate this (Loehr and Hanawalt, 1977; 1.3.4). The results presented here do not suggest a requirement for protein synthesis in the termination of replication, although it has not been demonstrated directly that the chromosome was completely replicated in chloramphenicol-treated cells.

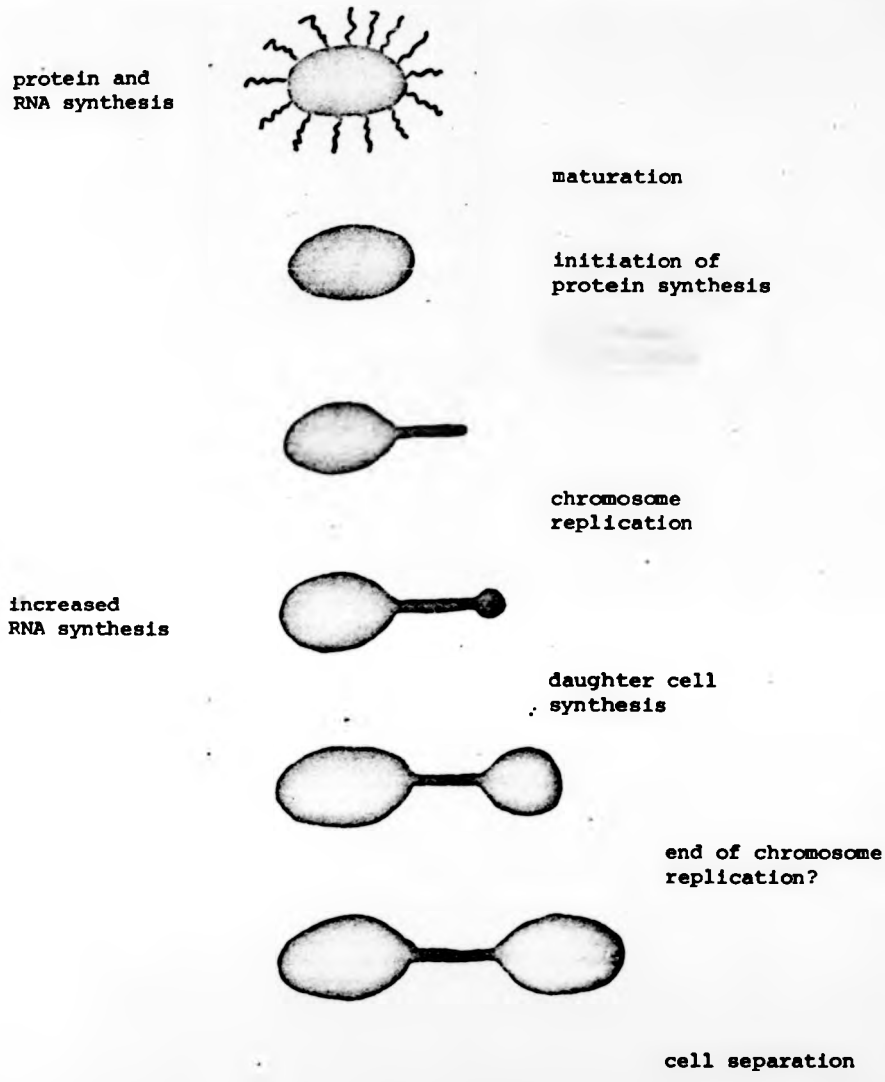


Fig. 5.11. Timing of biochemical events in the obligate differentiation sequence of R.vannielii swarm cells

CHAPTER 6

Transfer of the R.68·45 plasmid from E.coli to R.vannielii

6.1. Introduction

This chapter is concerned with the development of a genetic exchange system in R.vannielii, to allow fine structure gene mapping, and in the long term, the isolation of particular genes and regions of the chromosome of interest. It became clear that genetic analysis of the R.vannielii genome was crucial to an investigation of cellular morphogenesis and differentiation.

The genetics of R.vannielii had not been thoroughly investigated by previous workers. To date, no virulent or temperate phage, specific for R.vannielii has been isolated (C. S. Dow, personal communication). Genetic mapping by transduction analysis is therefore not feasible. The possibility of genetic transformation in R.vannielii has not yet been examined, but in the Rhodospirillaceae as a group, transformation has not been demonstrated (Saunders, 1978). Gene transfer mediated by the Gene Transfer Agent (GTA) has however been reported for R.capsulata (Marrs, 1974).

In plasmid-harbouring Rhodospirillaceae (e.g. R.sphaeroides), genetic conjugation through native plasmids has not been shown, although promiscuous plasmids of the 'P' incompatibility (incP) group may promote conjugation (Sistrom, 1977; Miller and Kaplan, 1978). In R.vannielii, although no native plasmids have been found (3.6), it may be possible to use the incP plasmids in the development of a genetic exchange system.

The multiple drug resistance, incP plasmid R.68·45 (a derivative of RP4), isolated by Haas and Holloway (1976) promotes gene transfer between species, and also between genera (Martinez and Clarke, 1975; Sistrom, 1977). The plasmid integrates at multiple sites in the chromosome, and so cannot be used for conventional marker mapping.

However, RP4-prime plasmids, containing fragments of E.coli DNA inserted in vitro, have been constructed, and these promote polarized chromosome transfer, perhaps due to the region of homology between the plasmid and the chromosome (Barth, 1979; Juillot and Boistard, 1979). Conventional gene mapping is then possible.

The transfer of R.68·45 from E.coli to R.vannielii was attempted with the aim of developing a genetic transfer system which would allow detailed analysis of the genome. The regions of the genome coding for nitrogen fixation or parts of the photosynthetic pathway and the genes involved in morphogenesis and differentiation, could be identified by this system, and then investigated in detail.

6.2. The occurrence of spontaneous antibiotic-resistant mutants in R.vannielii

Throughout all this work, R.vannielii expressing the 'simplified cell cycle' was used since cell counts obtained with this cell type were more accurate than with microcolonies of cells. The R.68·45 plasmid confers resistance to ampicillin (100 μg ml^{-1}), tetracycline (10 μg ml^{-1}) and kanamycin (40 μg ml^{-1}), and so initially the rate of occurrence of spontaneous resistance of R.vannielii to these antibiotics was tested.

An R.vannielii culture was grown to the late logarithmic phase (A_{540nm} ~ 3.5; cell count by Coulter counter analysis was found to be 6 x 10^8 cells ml^{-1}). Serial dilutions of the culture were spread onto plates containing i) PM alone, ii) PM + ampicillin (100 μg ml^{-1}), iii) PM + tetracycline (10 μg ml^{-1}), and iv) PM + kanamycin (40 μg ml^{-1}). No colonies appeared on any of the plates containing antibiotic after an incubation period of 8 days, whereas on PM alone, ~100 colonies grew at the 10^{-6} dilution, confirming that there were ~10^8 cells ml^{-1} in the initial culture. Consequently the frequency of spontaneous resistance

to any one of the antibiotics was less than 10^{-7}.

Polydiscs showed that R.vannielii was resistant to a low concentration of clindamycin and trimethoprim, and E.coli was sensitive to trimethoprim but resistant to clindamycin. A more precise experiment was undertaken to test the trimethoprim resistance of both bacteria. This showed that E.coli was sufficiently resistant to trimethoprim, at the concentration at which R.vannielii was resistant, to prevent the use of trimethoprim as a counter-selection against E.coli in conjugation experiments.

6.3. Isolation of antibiotic-resistant mutants of R.vannielii

6.3.1. Selection. Selection of an antibiotic-resistant mutant was achieved by spreading a dense culture of R.vannielii (10^8-10^9 cells) onto PM agar plates containing the particular antibiotic. Assuming that the mutation occurs with a frequency of 10^{-6} or lower, mutants should occur a few times on the plates with the highest cell density. The antibiotics used were nalidixic acid, rifampicin and streptomycin, over a concentration range of 10, 50 and 100 μg ml^{-1}. Serial dilutions of R.vannielii (5×10^{10} cells ml^{-1}) were spread onto the antibiotic plates, and duplicate plates were incubated anaerobically in the light (phototrophically) or aerobically in the dark (chemoheterotrophically). The results are shown in Table 6.1. No mutants arose on any of the plates incubated chemoheterotrophically, after an incubation of 8 days. The mutants obtained on phototrophically-incubated plates were maintained on selective agar plates. E.coli containing R.68.45 showed no spontaneous resistance to streptomycin at 50 μg ml^{-1}, so the streptomycin-resistant mutant of R.vannielii became the mutant of choice for conjugation experiments. The other mutants which were isolated may prove useful for later studies.

Table 6.1. Selection of antibiotic resistant mutants of *R.vannielii* in phototrophic culture

Antibiotic concentration	Colony number at each dilution 10^{-1}	10^{-2}	10^{-3}	Mutation frequency
rifampicin 10 μg ml^{-1}	>100	6	-	1.2×10^{-8}
nalidixic acid 10 μg ml^{-1}	>100	∿60	-	1.2×10^{-7}
streptomycin 10 μg ml^{-1}	>100	17	-	3.5×10^{-8}
streptomycin 50 μg ml^{-1}	24	1	-	2×10^{-9}

Original cell number = 5×10^{10} cells ml^{-1}

No mutants were detected which were resistant to rifampicin and nalidixic acid at 50 and 100 μg ml^{-1}, or streptomycin at 100 μg ml^{-1}.

A dense culture (5×10^{10} cells ml^{-1}) of the 'simplified cell cycle' expression, was serially diluted and plated onto PM agar plates containing each antibiotic, and incubated phototrophically (results above). Plates incubated chemoheterotrophically failed to develop distinct colonies. After 7 days' incubation the plates were scored for the appearance of antibiotic-resistant colonies. These were restreaked onto selective plates, and finally grown in liquid culture.

6.3.2. Mutagenesis. R.vannielii was treated with N-methyl-n'-nitro-N-nitrosoguanidine (NTG) to increase the frequency of mutation, using the method described by Miller and Kaplan (1978). A culture containing 2 x 10^8 cells ml^{-1} was incubated with 100 µg ml^{-1} NTG for 20 min. at 30°C. After washing, the cells were serially diluted, spread onto selective plates, and incubated phototrophically for 8 days. No colonies arose on any of the plates. It is possible that the conditions of incubation with NTG, while satisfactory for R.sphaeroides, were lethal for R.vannielii. Optimisation of the conditions for mutagenesis of R.vannielii could have been explored, but since mutants had been obtained by selection, this approach was not pursued.

6.4. Transfer of R.68.45 from E.coli to R.vannielii (wild type) and R.vannielii str^R

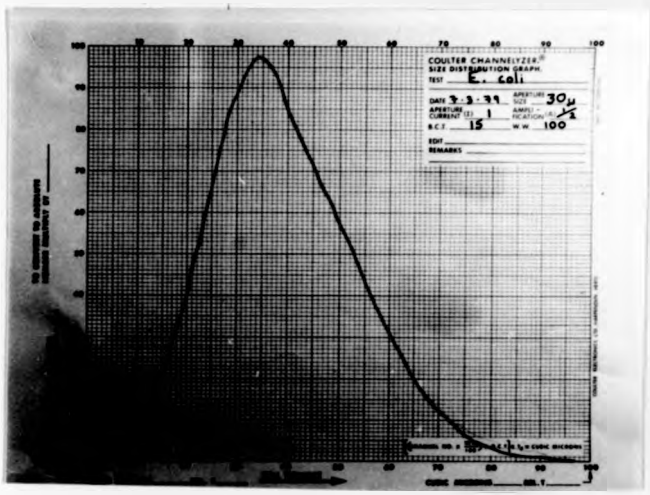
Initial conjugation experiments were done using wild type R.vannielii since the streptomycin-resistant mutant had not been isolated at that time. The strain E.coli C met^- [R.68·45] was used throughout the experiments. The literature records several procedures for bacterial conjugation. These include liquid mating (Miller and Kaplan, 1978), mating on solid media (Sistrom, 1977), patch mating on solid media (Tucker and Pemberton, 1979b) and filtration onto a filter followed by incubation on solid media (Ely, 1979). In this investigation the efficiencies of liquid and solid media matings were compared, and various modifications to the solid media mating technique were devised.

6.4.1. Liquid mating. In an initial conjugation in liquid culture, E.coli and R.vannielii cells were grown until the cell density of both cultures reached 10^8 cells ml^{-1}. The cell volume distribution

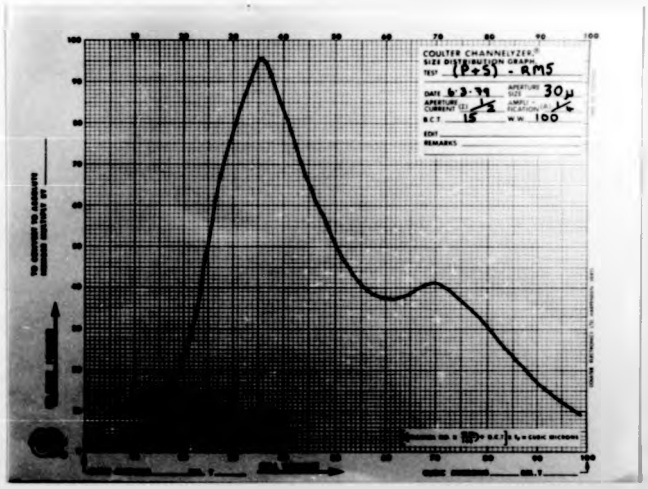
of R.vannielii shows the predominance of two cell types: the motile swarm cell and the double cell (Fig. 6.1). The cells were then harvested by centrifugation, washed and resuspended to 10^9 cells ml^{-1}. Equivalent amounts of each species were mixed and incubated for 5 hours at 30°C without shaking. Portions of the conjugation mixture were incubated phototrophically and chemoheterotrophically. Cultures were then serially diluted, plated onto non-selective PM medium and incubated for 18 hours, before the plates were overlaid with agar containing ampicillin (100 µg ml^{-1}). After incubation for 4 days, examination of the plates revealed the growth of red colonies at 10^{-1} and 10^{-2} dilutions, but none at 10^{-3}. The growth was spread evenly over the plate rather than being in distinct colonies. Moreover, growth was directed into the agar, away from the surface, perhaps to maintain an anaerobic environment. Growth from these plates was streaked onto PM plus ampicillin (100 µg ml^{-1}) plates. Restreaked colonies from the anaerobic set of matings showed no growth after 4 days incubation on the selective plates. Three of the four aerobic matings showed R.vannielii growth as well as some residual E.coli growth.

Restreaked R.vannielii transcipient colonies were tested for resistance to the non-selected antibiotics by plating on PM plus kanamycin or tetracycline. None of the colonies showed resistance to kanamycin or tetracycline, even though ampicillin resistance was maintained. Pure colonies of the R.vannielii exconjugants were difficult to obtain since there was often contamination with residual E.coli. However, repeated restreaking onto PM plus ampicillin plates was successful in producing pure cultures.

This liquid mating experiment showed that transfer of R.68·45 to R.vannielii did occur, but it was not possible to quantitate the



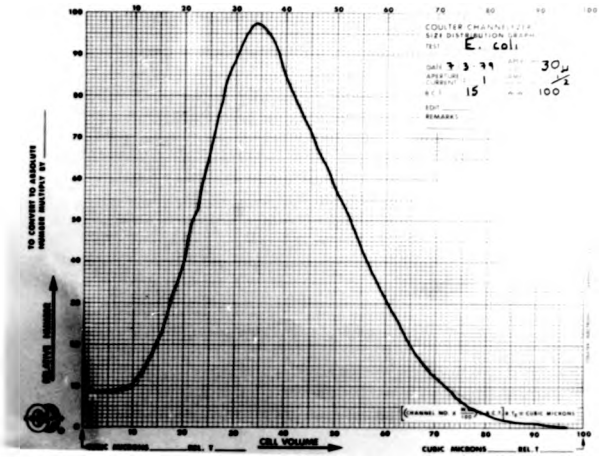
a) E.coli



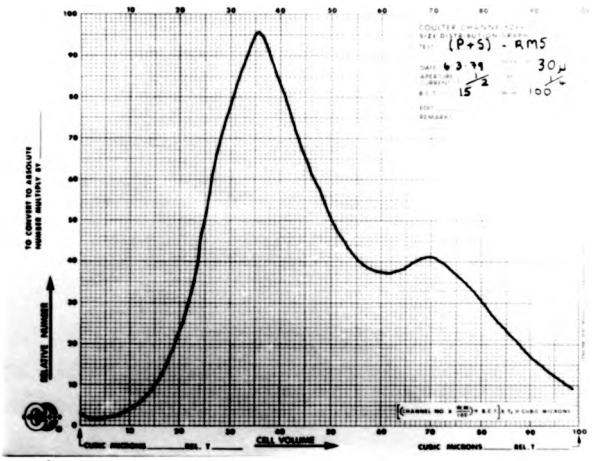
b) R.vannielii 'simplified cell cycle'

Fig. 6.1. Cell volume distribution of E.coli and R.vannielii 'simplified cell cycle' cells used in conjugation experiments.

After growth to 10^8 cells ml^{-1}, cell cultures were diluted in 'Isoton' and analysed in the Coulter counter and channelyzer (2.14).



a) E.coli



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frequency of transfer. Aerobic mating was more efficient than anaerobic, and kanamycin and tetracycline resistances were not expressed in ampicillin-resistant exconjugants of *R.vannielii*. This last point merits further investigation.

A second liquid conjugation experiment showed that equivalent numbers of donor and recipient cells promoted more efficient transfer than did either a $\frac{1}{10}$ or $\frac{1}{100}$ ratio of donor to recipient cell (Table 6.2). A further experiment in which kanamycin resistance was used as the selected marker, failed to detect any *R.vannielii* exconjugants, again suggesting that kanamycin resistance may not be expressed. The possibility that exconjugant *R.vannielii* becomes resistant to lower concentrations of antibiotic than *E.coli* has not yet been tested.

In order to test whether the whole of plasmid R.68·45, or just a part containing the ampicillin resistance gene was transferred to *R.vannielii*, a back-transfer experiment from *R.vannielii* [presumed R.68·45] to *E.coli* HB101 was designed. Conjugation was attempted in liquid medium, and equivalent and $\frac{1}{10}$ conc. *R.vannielii* cells were used. Since *E.coli* HB101 carries chromosomal resistance to streptomycin, streptomycin resistance could be used as a counter selection against *R.vannielii*. Table 6.3. shows that transfer of R.68·45 from *R.vannielii* to *E.coli* occurred at a higher frequency than transfer from *E.coli* to *R.vannielii*. The aerobic mating was slightly more efficient, but not significantly so. A $^{1}/10$ donor to recipient ratio increased the frequency of marker transfer in contrast to what was found for transfer in the reverse direction. Transfer of the complete plasmid occurred on average in 47% of the exconjugants carrying ampicillin resistance, implying that the plasmid

Table 6.2. Effect of donor cell number on the efficiency of plasmid transfer

Initial donor cell conc$^{n.}$ (cells ml^{-1})	Frequency of selected marker transfer per donor cell
1.7×10^{9}	2.05×10^{-3}
1.7×10^{8}	1.3×10^{-5}
1.7×10^{7}	7.0×10^{-5}

Recipient cell concentration = 2.4×10^{9} cells ml^{-1}

1 ml of both donor and recipient cells were mixed and incubated aerobically at 30°C for 5 hours. After recovery in PM medium for 5 hours, serial dilutions were plated onto selective media (PM + 100 µg ml^{-1} ampicillin), and the plates were incubated phototrophically for 5 days.

Table 6.3. Efficiency of back transfer of R.68.45 from R.vannielii amp^R to E.coli HB101 str^R

	Mating mixture	Frequency of phenotype per donor cell	
		amp^R str^R	kan^R,tet^R,amp^R,str^R
ANAEROBIC	equivalent cell numbers	5.5×10^{-3}	3.5×10^{-3}
	$\frac{1}{10}$ donor cell conc.	3.8×10^{-2}	8.8×10^{-3}
AEROBIC	equivalent cell numbers	8.8×10^{-3}	6.4×10^{-3}
	$\frac{1}{10}$ donor cell conc.	5.1×10^{-2}	1.6×10^{-2}

may fragment on transfer. R.vannielii can maintain the intact plasmid, but fails to express kanamycin and tetracycline resistance.

More evidence was sought for the ability of R.vannielii to harbour an intact R.68·45 plasmid by the demonstration that plasmid DNA isolated from both E.coli [R.68.45] and R.vannielii amp^R could be detected on a 0.8% agarose gel (Fig. 6.2). The R.vannielii plasmid band is very faint and slightly above that of E.coli, but both tracks show extensive nicking of the plasmid. Further purification of the plasmid was unsuccessful, and so there is not conclusive evidence that the whole of R.68·45 was transferred into R.vannielii cells.

6.4.2. Mating on a solid medium. Initially, the solid mating technique described by Sistrom (1977) was attempted. Donor and recipient cells were mixed at a density of 10^9 ml^{-1}, and 20 µl aliquots were spotted onto PM agar plates. After incubation (either phototrophically or chemoheterotrophically) at 30°C for 5 hours, the cells were harvested in phosphate buffer (2.2), allowed to recover in PM media for 8 hours, and spread on PM plus ampicillin (100 µg ml^{-1}) plates. After 8 days' incubation no red exconjugants were found on the plates. Yellowed growth of R.vannielii was observed, but when this was restreaked onto selective plates, the cells failed to grow well. Consequently this method of conjugation could not be used for R.vannielii. This could be because conjugation did not occur, or because of technical difficulties such as the inefficiency of cell harvesting.

The difficulty in ensuring R.vannielii exconjugants were free of residual E.coli contamination highlighted the usefulness of R.vannielii mutants carrying a chromosomal antibiotic resistance which could be used in counter-selection against E.coli. Other

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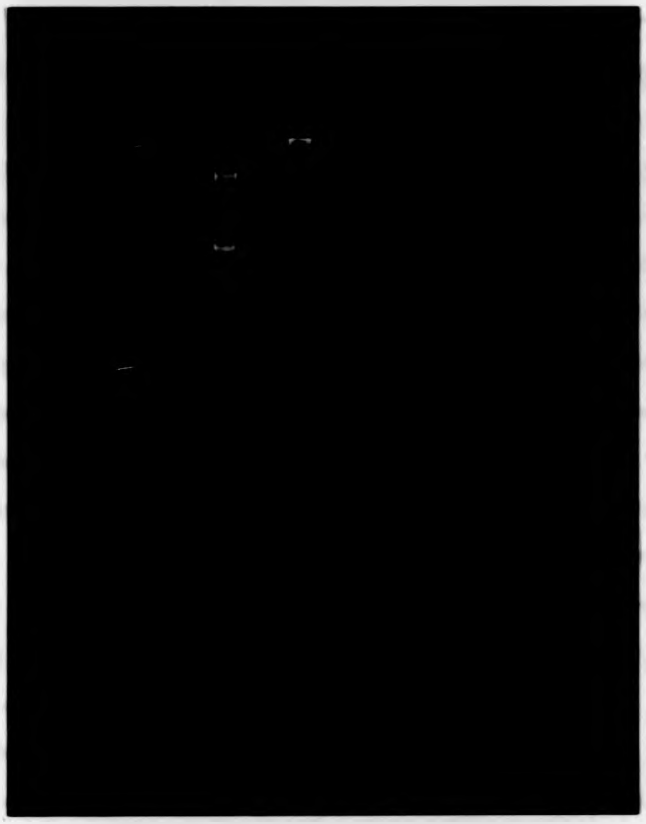
3 2 1



◀ Fragment of plasmid?

Fig. 6.2. Gel electrophoresis of plasmid DNA from E.coli and and R.vannielii after conjugation experiments

Plasmid DNA was isolated from E.coli [R.68.45] and R.vannielii amp^R cells by the cleared lysate procedure (2.25.1). DNA was electrophoresed in 0.8% (w/v) agarose at 35 v overnight.

1. E.coli [R.68.45]
2. R.vannielii Amp^R
3. λ DNA restricted with EcoRl and HindIII

Intact R.68.45 should have banded near the top of the gel.

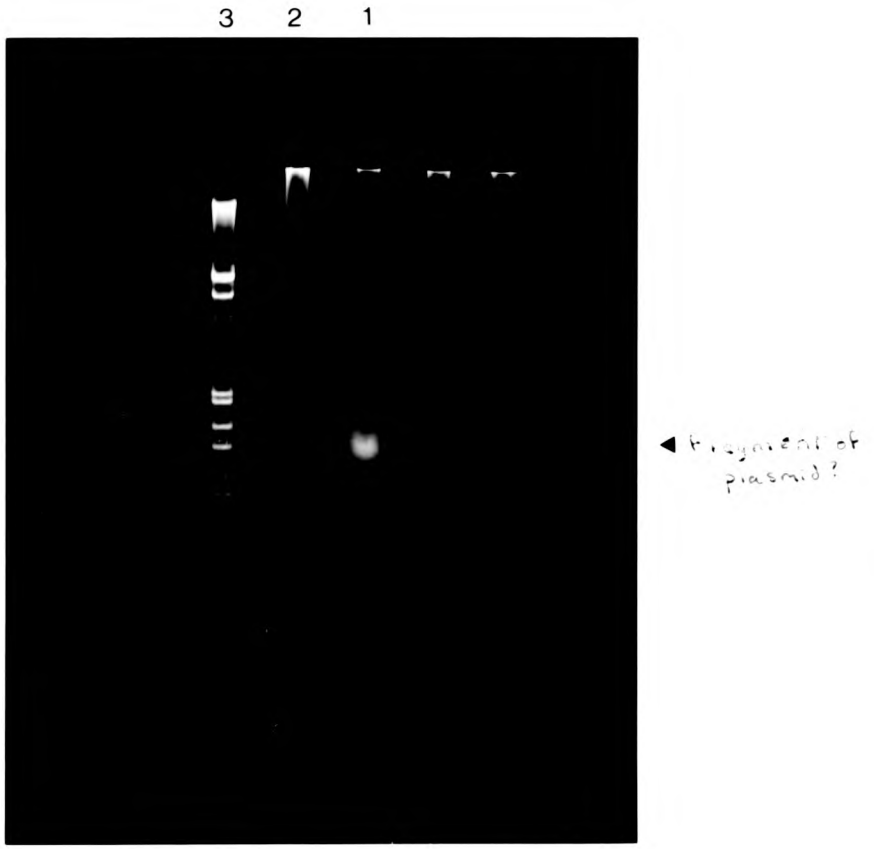


Fig. 6.2. Gel electrophoresis of plasmid DNA from E.coli and and P.vannielii after conjugation experiments

Plasmid DNA was isolated from E.coli [P.68.45] and P.vannielii amp^R cells by the cleared lysate procedure (2.25.1). DNA was electrophoresed in 0.8% (w/v) agarose at 35 v overnight.

1. E.coli [P.68.45]
2. P.vannielii Amp^R
3. λ DNA restricted with EcoP1 and HindIII

Intact R.68.45 should have banded near the top of the ge

possible ways of eliminating E.coli are treatment with antibiotics in conditions which allow the growth of E.coli but not R.vannielii, or the use of an E.coli-specific virulent phage which does not affect R.vannielii. A streptomycin resistant mutant of R.vannielii (resistant to 50 µg ml^{-1} streptomycin) was obtained by selection (6.3.1), and was used in all further conjugation experiments.

Since the mating on solid medium had been unsuccessful with wild type R.vannielii, the technique was modified for conjugation experiments with the streptomycin resistant mutant. In the method of Sistrom (1977), 20 µl aliquots had been spotted onto a 9 cm petri dish, and cells were harvested in buffer after the incubation period. Since a large volume (1 ml) of buffer was required to recover the cells from the dish, the initial dilution factor was already 50. Moreover this method did not allow consistent recovery of the cells from the dish.

Two alternative methods were devised:-

a) PM agar was cast into Bijou bottles (5 ml), to provide a horizontal surface,

b) PM agar was cast in 3 cm petri dishes.

In both these cases 20 µl aliquots of conjugation mixture was applied. Cells were harvested in 200 µl of buffer, which ensured only a 10 fold dilution, and recovery of the cells was more efficient since the total surface area of agar to be harvested was less than in the larger petri dish. The same cell cultures were allowed to conjugate in liquid medium so that the efficiencies of the methods could be compared. After a 5 hour incubation, followed by a recovery period, serial dilutions of the exconjugants were spread onto PM plus ampicillin (100 µg ml^{-1}) plates. The results are shown in

Table 6.4. The small petri dish method proved to be the more efficient by a factor of 100 over the Bijou bottle technique. Although this particular liquid mating was not very successful, when the solid mating on a petri dish was compared to previous matings in liquid, they were still marginally more efficient. Another important factor was the ease of manipulations with the small petri dish compared with the Bijou bottle. This latter method was less efficient due to the poor recovery of cells in the buffer. The method of choice in all future solid medium conjugation experiments is the one employing the small petri dish.

6.5. The effect of the length of conjugation time on the frequency of transfer of ampicillin resistance from E.coli [R.68·45] to R.vannielii str^R

The mating was done aerobically on solid medium in a small petri dish, and a slightly different method was used for assessing the results. To minimize the number of plates required, 20 µl of each serial dilution were spotted onto a single selective plate (PM plus ampicillin 100 µg ml^{-1}), so that the whole concentration range of one test was on one plate. After incubation at 30°C under phototrophic conditions for 4-7 days, microcolonies in each spot were counted. This method did not prove to be efficient for R.vannielii, since often the cells were grouped in a thick ring around the edge of the spot, and were difficult to count. The results are shown in Table 6.5. A 10 hour conjugation time seems to be the most efficient since the frequency of marker transfer decreases on either side of this time. However a large, significant difference in efficiency between the various conjugation times was not apparent. Further experiments are necessary to investigate the least conjugation time required, since the results obtained here are very variable.

Table 6.4. Efficiency of selected marker transfer under different mating regimes.

Mating method	Frequency of selected marker transfer per donor cell
liquid medium	$<1.0 \times 10^{-5}$
solid medium (Bijou bottle)	3.2×10^{-5}
solid medium (3 cm petri dish)	3.2×10^{-3}

Initial concentration *E.coli* = 6×10^{8} cells ml^{-1}

" " *R.vannielii* = 2.3×10^{9} cells ml^{-1}

Equivalent volumes of donor and recipient cells were mixed. Liquid matings were incubated aerobically at 30°C. For solid medium conjugation, 20 µl mating mixture was spotted onto PM agar either in a Bijou bottle, or in a 3 cm petri dish. These were incubated aerobically at 30°C. After incubation for 5 hours cells were harvested in 200 µl buffer, allowed to recover in PM media for 8 hours, and serially diluted onto selective plates. Ampicillin resistance was the selected marker.

Table 6.5. Effect of conjugation time on the frequency of selected marker transfer from *E.coli* R.68.45 to *R.vannielii* str^R

Conjugation time (hrs.)	Frequency of marker transfer per donor cell
5	5×10^{-5}
10	1.86×10^{-4}
20	5×10^{-6}
24	1.25×10^{-4}

Initial concentration *E.coli* = 8×10^8 cells ml^{-1}

" " *R.vannielii* = 2.5×10^8 cells ml^{-1}

Equivalent numbers of donor and recipient cells were mixed, and 20 µl was spotted onto PM agar in a 3 cm petri dish and incubated aerobically at 30°C. After the conjugation time, cells were harvested in 200 µl buffer, allowed to recover in PM media for 8 hours, and serially diluted. 20 µl of each dilution was spotted onto a selective plate (PM plus ampicillin 100 µg ml^{-1} and streptomycin 50 µg ml^{-1}), and microcolonies were counted after 4-7 days.

6.6. Discussion.

Transfer of the R.68·45 (inc P group) plasmid from E.coli to R.vannielii has been clearly demonstrated, since the frequency of transfer of antibiotic resistance associated with the plasmid is too high to be explained by bacterial or plasmid mutation. Consequently, conjugation offers the best opportunity for in vivo genetic manipulation in R.vannielii, although chromosomal mobilization has not yet been demonstrated.

The initial problem associated with the elimination of E.coli after conjugation was effectively overcome by the use of a chromosomal streptomycin-resistant mutant of R.vannielii. Aerobic matings were more efficient than anaerobic, perhaps because of a requirement for E.coli growth in conjugation. Under anaerobic conditions conjugation did occur, although at a reduced frequency. This is in contrast to the findings of Miller and Kaplan (1978), who showed with R.sphaeroides that no conjugation occurred under phototrophic conditions. The plasmid RP4 has been transferred to anaerobic Bacteroides spp (Burt and Woods, 1976), indicating that E.coli can express transfer functions under anaerobic conditions.

In early experiments the liquid mating technique proved to be more efficient than mating on a solid medium, contrary to the findings of both Miller and Kaplan (1978) and Sistrom (1977), that in R.sphaeroides conjugation on agar was far more efficient. In later experiments with R.vannielii, when the solid medium method was modified, a marked improvement in the efficiency of conjugation was obtained, and this modified method should be employed in further experiments.

Ampicillin resistance was the only plasmid-borne marker to be

expressed in R.vannielii, even although the entire plasmid had been transferred. Complete transfer was demonstrated by the ability of R.vannielii [R.68·45] to transfer all three plasmid-borne resistances back into E.coli, although confirmation of this by the comigration in an agarose gel of plasmid DNA from R.vannielii with that from E.coli was not conclusive. The activity of β-lactamase (the enzyme involved in ampicillin resistance) should be higher in R.vannielii [R.68·45] than in the wild type, but this has not yet been tested.

The inability of tetracycline and kanamycin resistance to be expressed in R.vannielii reflects some inherent physiological property of the R.vannielii cell. Miller and Kaplan (1978) have found that R.sphaeroides transcipients carrying RP4 did not express ampicillin resistance whereas they expressed and maintained tetracycline and kanamycin resistance. C.crescentus carrying R.68·45 expressed kanamycin and tetracycline resistances but whether or not plasmid-borne ampicillin resistance was expressed could not be determined since wild type C.crescentus was ampicillin resistant (Ely, 1979).

Future work with R.vannielii should be directed towards determining whether or not R.68·45 is able to mobilize the R.vannielii chromosome. To do this, chromosomal markers, usually auxotrophic markers, are required. Previous workers have experienced difficulty in isolating stable auxotrophic mutants of R.vannielii, and so at the present time it is not possible to test if chromosomal mobilization occurs.

Since R.68·45 has multiple insertion points on the chromosome, its usefulness in genetic transfer experiments with R.vannielii is limited. However structural modifications to the plasmid may overcome this problem. Dénarié et al. (1977) have examined the potential

for genetic analysis of incP plasmids carrying the temperate bacteriophage Mu. Mu has a powerful insertion system, but unfortunately it has a restricted host range, being limited to the Enterobacteriaceae. However, if inserted into the incP plasmids its host range is greatly extended. The insertion of RP4-Mu plasmids into Rhizobium and Pseudomonas has been demonstrated (Faelen et al., 1977). Since it has been shown that R.68·45 can be maintained in R.vannielii it should be possible in future genetic studies to use the RP4:Mu plasmid as a tool in the genetic analysis of this organism.

Another modification of incP plasmids was that of Barth (1979) and Juillot and Boistard (1979) which was mentioned in 6.1. They inserted a restriction fragment of E.coli DNA into the plasmid by ligation, and selected for a plasmid with the insert, which had lost resistance to one of the antibiotics. This recombinant plasmid was then transformed into E.coli and conjugated into other strains. Chromosomal mobilization was found to be polarized, presumably because of the region of homology now present between chromosome and plasmid. This technique could be adapted to R.vannielii since restriction analysis of the genome has been developed alongside this work (Chapter 7). This technique could allow analysis of specific genes such as those for nitrogen fixation, and those involved in the control of morphogenesis and differentiation.

CHAPTER 7

Experiments directed towards the genetic mapping of R.vannielii

7.1. Introduction

The development of a genetic system by conjugation in R.vannielii is potentially useful for future genetic analysis of the bacterium. However, as well as traditional genetic mapping, new techniques are now available as a result of the recent developments of recombinant DNA construction and gene cloning (Helling and Lomax, 1978). Restriction endonuclease mapping has been successfully applied to the analysis of many genomes, particularly those of viral origin, e.g. SV40 (Danna et al., 1973), and more recently has been used to 'fingerprint' Rhizobium mutants (Mielenz et al., 1979).

The construction of recombinant DNA molecules made up of the DNA sequence of interest and a plasmid or phage 'vector' DNA, and subsequent transformation of E.coli cells (Taketo, 1972), allows the expression of that sequence in E.coli, and amplification of the gene product(s) involved (see for example Cohen, 1975). This basic technology has been used to great effect with both prokaryotic and eukaryotic gene sequences (e.g. the mammalian growth hormone gene, Seeburg et al., 1978), enabling both the gene in question and its regulation to be examined in detail.

The applicability of these methods to the study of R.vannielii is twofold. The first approach utilizes existing E.coli strains as cloning vehicles in which to study the expression of specific R.vannielii genes. This would involve the preparation of specific radioactive 'probes' (mRNA or cDNA of a particular sequence) with which to screen colonies resulting from the cloning of total DNA fragments from R.vannielii in E.coli. Colonies showing hybridization with the radioactive probes would be selected, and grown in quantity,

allowing the amplification of the selected sequence for studies of its structure and function. This method of gene analysis would be useful for studying the ribosomal RNA genes, nitrogen fixation genes, the genes for RuBP carboxylase enzyme,or flagellin protein, and the origin of replication. Genes of particular interest in R.vannielii are those involved in the regulation of morphogenesis and differentiation, but the genes mentioned above are more immediately amenable to study, and consequently have been examined initially.

The second possible approach is to use R.vannielii itself as a cloning vehicle, and amplify specific homologous gene sequences carried on a plasmid or phage. This is an attractive proposition since it is better to study the regulation of a gene in its 'usual' environment, that is, an R.vannielii gene in R.vannielii, rather than in E.coli. A fundamental requirement is for a phage or plasmid vector DNA into which the particular gene of interest may be inserted. As mentioned in the introduction (1.7) and in the previous chapter, this requirement is difficult to fulfil at the present time, as no suitable vectors for R.vannielii have been described.

The studies described in this chapter utilized the first approach described above, and although the work is still in its preliminary stages, it is anticipated that these experiments will lay the foundation for a more detailed genetic analysis of R.vannielii. Three specific regions of the genome were chosen:- the ribosomal RNA genes, the origin of replication, and the nitrogen fixation genes. Each of these will be considered individually, after a discussion on the use of restriction enzyme digestion in the analysis of the R.vannielii genome.

7.2. Restriction endonuclease mapping of the R.vannielii genome

7.2.1. Introduction. The discovery of restriction endonucleases has revolutionised molecular biology on an unprecedented scale (Smith, 1979), and has led to exciting discoveries in recent years (Jeffreys and Flavell, 1977). Restriction endonucleases are enzymes which cleave double-stranded DNA at specific sites. Type I enzymes recognize a particular DNA sequence and cleave the DNA elsewhere, whereas Type II enzymes recognize and cleave at the same site, and so the latter have the greater potential for the manipulation of DNA. Restriction enzymes may recognize sequences 4 or 6 nucleotides in length, and within that sequence each base may be specific or only a purine or pyrimidine base may be required (Nathans and Smith, 1975).

In this study, enzymes which recognize hexanucleotide sequences were used, since it was likely that these would occur less frequently in the DNA, and hence less fragments would be generated, making analysis easier. Three enzymes only were chosen, since further experiments utilising the resulting DNA fragments were planned. The enzymes were :-

a) *Eco* Rl which cleaves at 5' G↓A A T T C 3'
(Hedgepeth *et al.*, 1972)

b) *Hin* dIII which cleaves at 5' A↓A G C T T 3'
(Old *et al.*, 1975)

c) *Bam* Hl which cleaves at G↓G A T C C
(Roberts *et al.*, 1977)

7.2.2. Isolation of high molecular weight DNA. The DNA prepared for restriction enzyme analysis should have a high molecular weight, since the presence of random breaks in DNA leads to a heterogeneous mixture of fragments after enzyme digestion. It is difficult to

avoid some shearing in large-scale preparations of DNA, but precautions were taken to reduce this to a minimum.

The DNA was extracted from large-scale (2-5 litres) cultures of cells in the exponential phase of growth (A_{540nm} ~ 2.0-2.8), by the method described in 2.21.2., and was extensively dialysed against 10 mM TRIS-HCl, pH 8 containing 1 mM EDTA. The resulting high molecular weight DNA was difficult to redissolve, since it did not rehydrate readily, and often gentle resuspension overnight was necessary.

An estimate of the size of the DNA preparations was made on a 0.6%(w/v) agarose gel (Fig. 7.1). Phage lambda (λ) DNA digested with Eco R1 and Hin dIII and undigested λ DNA were used as molecular weight markers (2.29.3). The average size of the DNA was 125 Kb, although some smaller fragments were also present. A more accurate estimate of the very high molecular weight DNA which did not enter the gel could be made by alkaline sucrose density-gradient centrifugation. This was thought to be unnecessary, since a qualitative estimate was sufficient.

7.2.3. Restriction endonuclease digestion of R.vannielii DNA. For all restriction enzyme assays, DNA was resuspended in 10 mM TRIS pH 7.4 containing 1 mM EDTA. The assay mixture was prepared as described in 2.28, with approximately 5 µg DNA per assay. Digestion usually proceeded overnight at 37°C, to minimize the amount of enzyme required, and to ensure complete digestion. The reaction was stopped by heating the sample at 65°C for 5 min., and the DNA fragments were separated by electrophoresis on agarose gels, alongside molecular weight markers (2.29.3).

A representative Eco R1 restriction of R.vannielii DNA is shown in

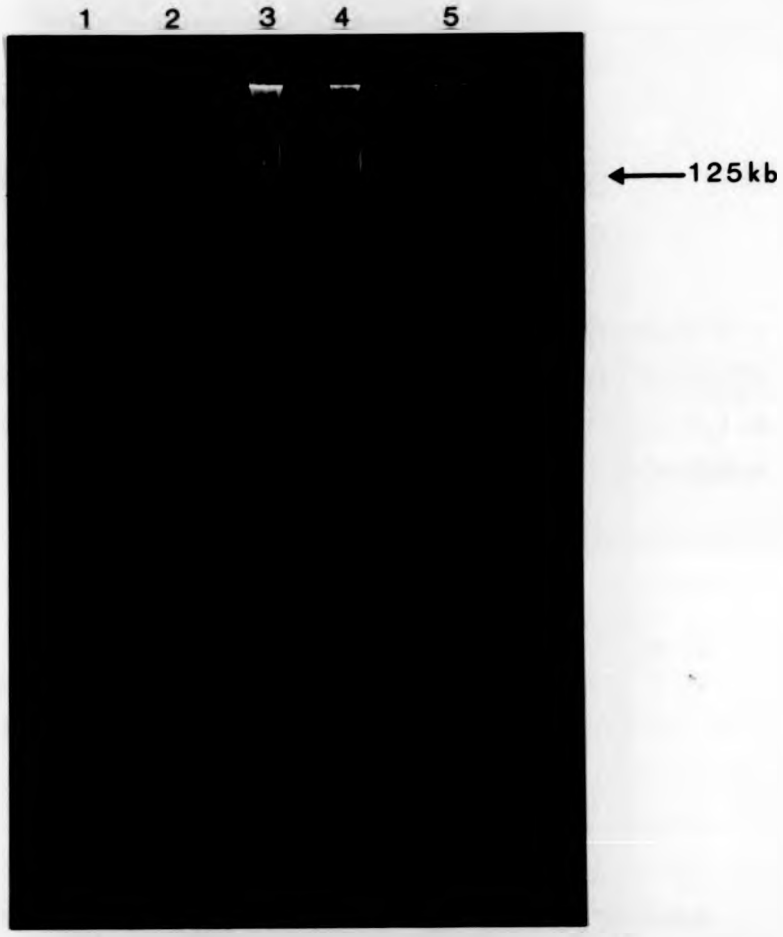


Fig. 7.1. Gel electrophoresis of high molecular weight R.vannielii DNA prepared for restriction enzyme analysis.

DNA was prepared as described in 2.21.2., and electrophoresed through 0.6% (w/v) agarose at 35 v overnight.

1. λ DNA restricted with EcoRl and HindIII
2. intact λ DNA
3.-5. R.vannielii DNA

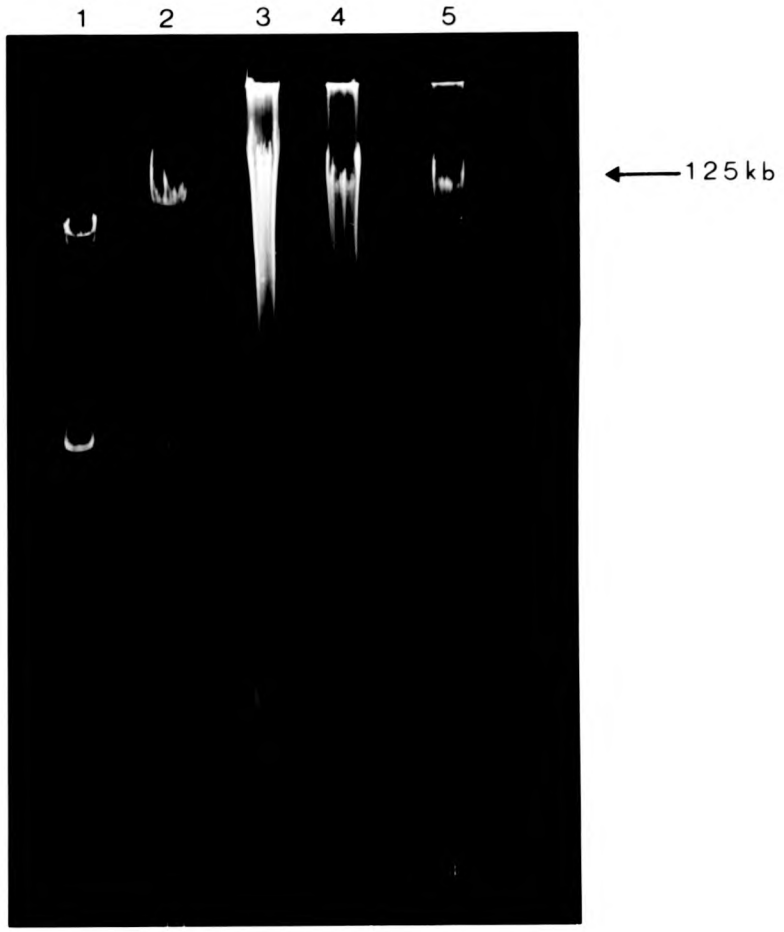


Fig. 7.1. Gel electrophoresis of high molecular weight P.vannielii DNA prepared for restriction enzyme analysis.

DNA was prepared as described in 2.21.2., and electrophoresed through 0.6% (w/v) agarose at 35 v overnight.

1. λ DNA restricted with EcoP1 and HindIII
2. intact λ DNA
3.-5. P.vannielii DNA

Fig. 7.2. Gel electrophoresis of EcoRl-restricted R.vannielii DNA

R.vannielii DNA was restricted with EcoRl overnight at 37°C (2.28), and the resulting fragments were electrophoresed in 1.5% (w/v) agarose at 75 v for 4 hours. λ - EcoRl restriction fragments were used as molecular weight markers.

1. λ DNA (1 µg), undigested
2. λ DNA restricted with EcoRl
3. R.vannielii DNA (2 µg), undigested
4. R.vannielii DNA (3 µg) restricted with EcoRl
5. R.vannielii DNA (5 µg) restricted with EcoRl
6. R.vannielii DNA (3 µg) and λ DNA (1 µg) restricted with EcoRl

Arrows refer to distinct bands in tracks 4 and 5

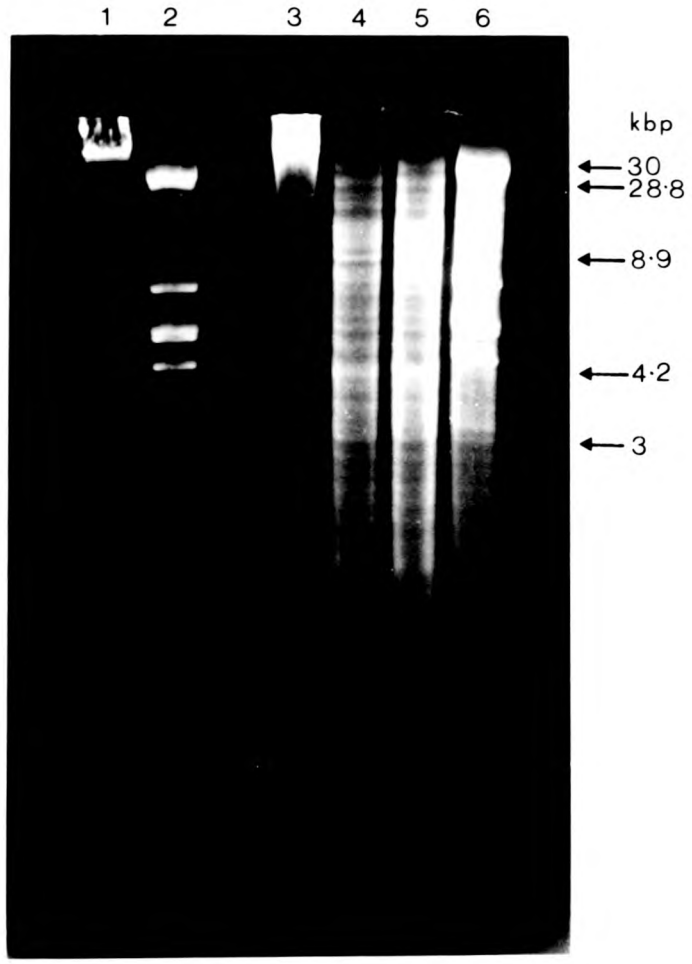

1
2
3
4
5
6
kbp
30
28·8
8·9
4·2
3

Fig. 7.2. Approximately 20 clear bands were seen in the digest, but others were present that were indistinguishable. The molecular weights of some fragments were determined by comparison with the Eco Rl fragments of λ DNA. λ DNA restricted within the R.vannielii DNA sample showed the 6-band pattern which is characteristic of a complete λ digest (Thomas and Davis, 1975), indicating that the R.vannielii DNA was also completely digested.

Fig. 7.3. shows the results of a Hin dIII restriction of R.vannielii DNA, electrophoresed through 0.6% (w/v) agarose using the vertical gel system (2.29.2). The use of a vertical gel and a lower agarose concentration gave better resolution of the many bands seen in the Hin dIII digestion. The major fragments in the Hin dIII digest were assigned molecular weights (Fig. 7.3).

An attempted Bam Hl restriction of R.vannielii DNA was electrophoresed in 1.5% (w/v) agarose using the horizontal gel system and the result is shown in Fig. 7.4. Although there was overloading of DNA on this gel, there was no restriction of the R.vannielii DNA. λ DNA included in the R.vannielii DNA restriction mixture (lane 6) showed the same fragments as the λ control (lane 3), suggesting that the activity of Bam Hl was not inhibited by factors in the R.vannielii DNA preparation. This lack of cleavage of R.vannielii DNA with Bam Hl was found in 6 separate enzyme assays.

7.2.4. Conclusions. Digestion of R.vannielii DNA with either Eco Rl or Hin dIII gave rise to definite patterns of restriction fragments, although the fragments were too many in number to classify each individually. Nonetheless the restriction patterns were of great use for in vitro hybridization studies, enabling specific fragments to be identified and later isolated for further investigation.

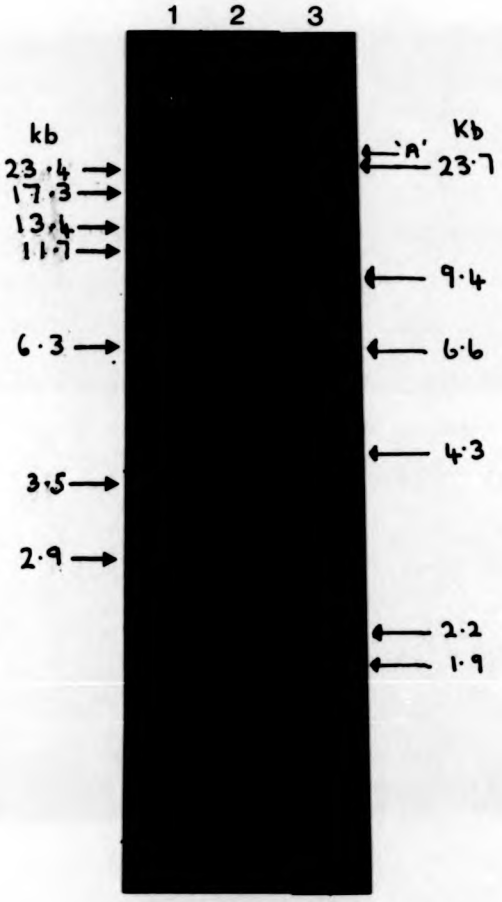
Fig. 7.3. Gel electrophoresis of *R.vannielii* DNA restricted with *Hind*III

DNA was restricted with *Hind*III overnight at 37°C (2.28), and the resulting fragments were electrophoresed in 0.6% (w/v) agarose at 35 v overnight.

1. *R.vannielii* DNA (2 µg) restricted with *Hind*III
2. " " (4 µg) " " "
3. λ DNA (2 µg) " " "

Band 'A' may be a partial digestion product. The other bands in track 3 were calibrated against an *Eco*R1 digest of λ DNA in the same gel.

Arrows on left refer to distinct bands in track 2.


1
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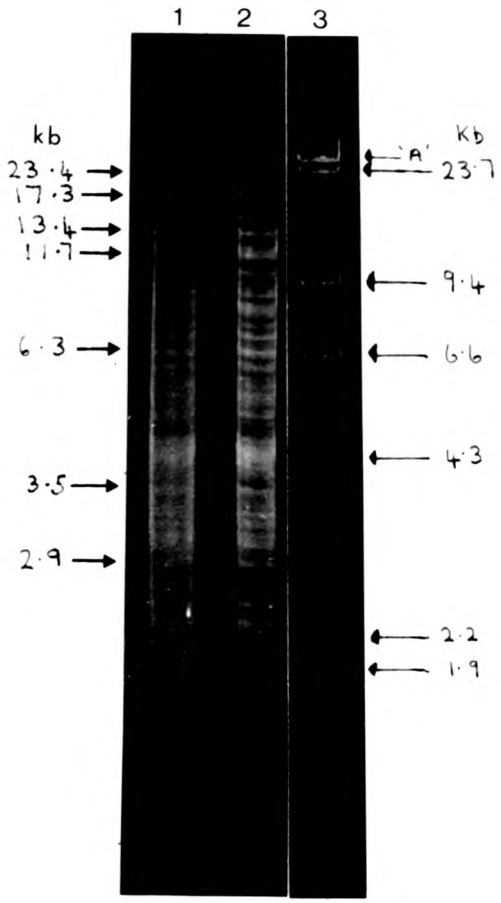
3
kb
23·4
17·3
13·4
11·7
6·3
3·5
2·9
Kb
'A'
23·7
9·4
6·6
4·3
2·2
1·9


1
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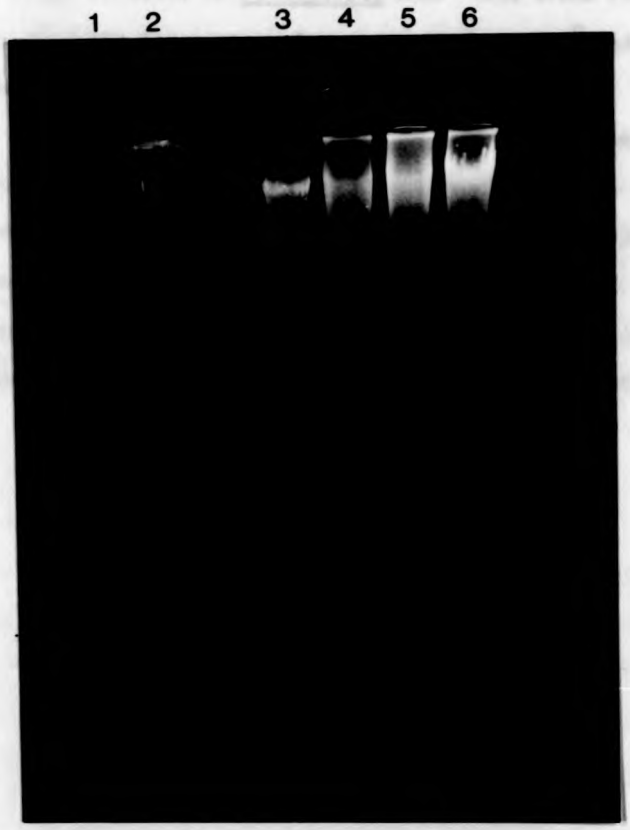
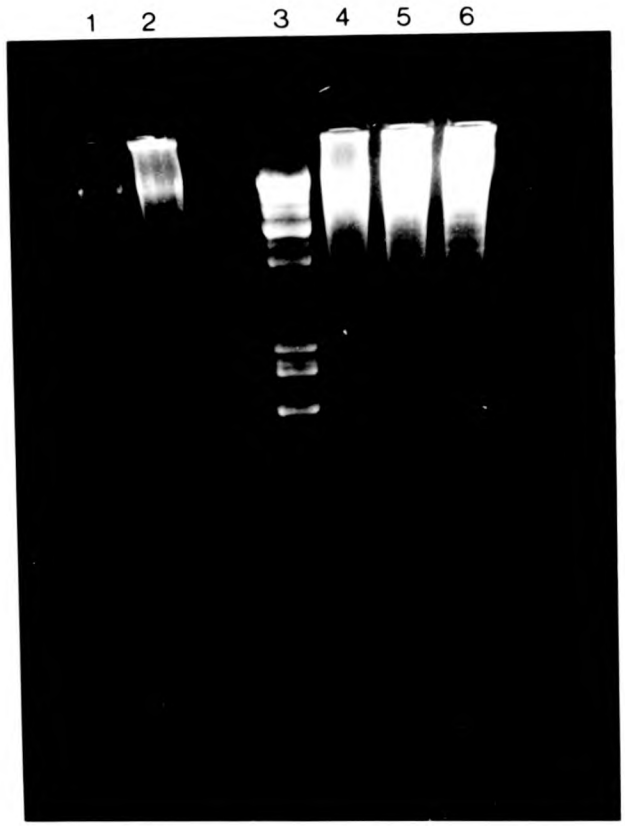
3
kb
23·4
17·3
13·4
11·7
6·3
3·5
2·9
Kb
'A'
23·7
9·4
6·6
4·3
2·2
1·9

Fig. 7.4. Gel electrophoresis of Bam Hl-restricted R.vannielii DNA.

R.vannielii DNA was restricted with Bam Hl overnight at 37°C (2.28) and the resulting fragments electrophoresed in 1.5% (w/v) agarose at 75 v for 4 hours.

1. λ DNA (1 μg)
2. native R.vannielii DNA (3μg)
3. λ DNA restricted with Bam Hl


1
2
3
4
5
6


1 2 3 4 5 6

The fact that Bam Hl did not cleave R.vannielii DNA can be interpreted in one of two ways. The first possibility is that there are no Bam Hl sites on R.vannielii DNA. This seems highly unlikely in view of the large size of the genome (2.1×10^9 daltons), and the enzymes' GC-rich recognition site.

An alternative explanation is that Bam Hl recognition sites are present on the DNA, but they are modified in some way to prevent restriction. The most usual modification is methylation of certain bases in the recognition sequence, protecting the DNA from restriction (Arber, 1974). This alternative is very likely, but conclusive evidence of methylation in R.vannielii DNA has not yet been obtained.

7.3. The ribosomal RNA genes

7.3.1. Introduction. The absence of viable mutants in the ribosomal RNA (rRNA) genes of E.coli made early studies of the genes difficult, but the development of merogenotes allowed the effects of gene dosage to be examined. Subsequently, rRNA became the subject of intensive investigation with the advent of sophisticated DNA-RNA hybridization technology. The main reason for this was the fact that rRNA comprises more than 90% of total cellular RNA, and so large quantities of RNA may be prepared easily. On the other hand, most individual mRNA molecules represent less than 0.1% of total RNA, making their purification very difficult. A secondary reason for studying rRNA is that it provides a useful model system for the investigation of transcriptional control of gene function. Most bacterial RNA consists of two molecules which are distinguished by their sedimentation properties: the first is 16s rRNA (MW 0.36×10^6 daltons) and the second is 23s rRNA (MW 1.1×10^6 daltons). However, Marrs and Kaplan (1977) demonstrated that in R.sphaeroides, 23s rRNA is a precursor to the stable 16s and 14s rRNA molecules, suggesting that the 23s rRNA molecule may not be required intact for ribosome function.

Data is already available on the rRNA genes of many organisms, particularly E.coli (Pace, 1973; Nomura et al., 1977). The rRNA genes in E.coli are present in multiple copies (Yankofsky and Spiegelman, 1962), this redundancy presumably having arisen to supply a large quantity of rRNA to rapidly-growing cells. The basic structure of the rRNA operon was shown to consist of:-
promoter - 16s rRNA gene - spacer - tRNA gene(s) - 23s rRNA gene - 5s RNA gene - distal tRNA gene(s) (Lund et al., 1976).

The aim of the present study was to locate the specific DNA

fragments which contained the rRNA genes in order to establish the distribution of these genes in the genome. The technique used was that developed by Southern (1975) which involves the hybridization of radioactive rRNA 'probes' to DNA fragments immobilized on a nitrocellulose filter. The method had been previously shown to be sensitive and accurate in detecting gene sequences in many biological systems.

7.3.2. Preparation of radioactive 16s rRNA and 23s rRNA 'probes'. Hybridization experiments require RNA of high specific activity (10^6 cpm μg^{-1}), and it was found that *in vivo* labelling of cellular RNA with ^{32}P-orthophosphate failed to provide such active material. Consequently, cellular rRNA was first purified, and then labelled at the 5' end with γ-^{32}P-ATP, using the enzyme polynucleotide kinase (Maizels, 1976; 2.30).

One litre of 'simplified cell cycle' *R.vannielii* cells were grown to an A_{540nm} of 3.5, and then harvested by centrifugation. Nucleic acids were extracted (2.21.1), and fractionated on 5-20% (w/v) neutral sucrose gradients in an MSF 65 centrifuge for 6 hr. at 190,000 g (2.22). The gradients were analysed in an Isco density gradient fractionator, and a representative profile is shown in Fig. 7.5. The peak of 23s rRNA should be greater than that for 16s rRNA if the proportions of the two rRNA's are similar in *R.vannielii* and *E.coli*. This discrepancy could be due in part to breakdown of the 23s rRNA.

Peak fractions from the gradient were pooled, and precipitated with 2 volumes of ethanol. Resuspended RNA was further purified by re-centrifugation in 5-20% (w/v) neutral sucrose gradients as described above. The A_{260nm} profile of these gradients is shown in Fig. 7.6.

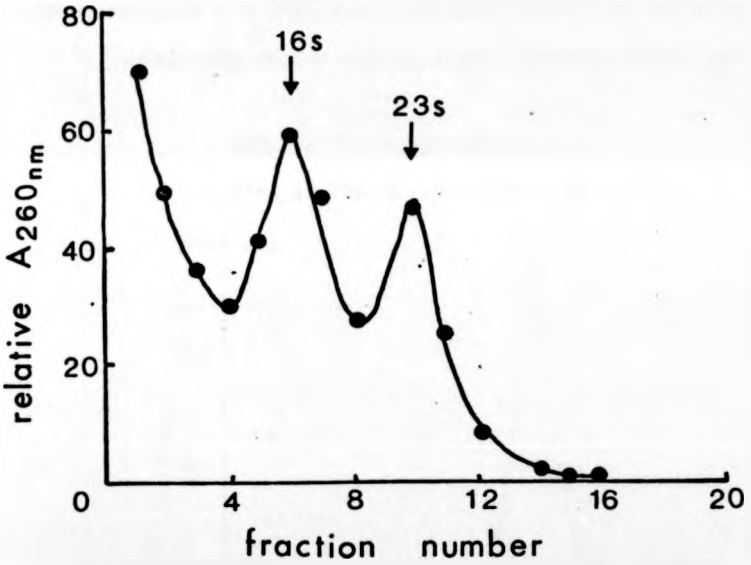


Fig. 7.5. A_{260nm} profile of total nucleic acid from R.vannielii fractionated on a sucrose gradient.

Sedimentation was from left to right. Units of absorbance are relative to a base line of 0.1 units/ml.

Total nucleic acids were fractionated on a 5-20% neutral sucrose gradient in TLES buffer (2.3.e)), for 6 hours at 190,000 g (2.22). Fractions (0.6 ml) were collected in an Isco density gradient fractionator.

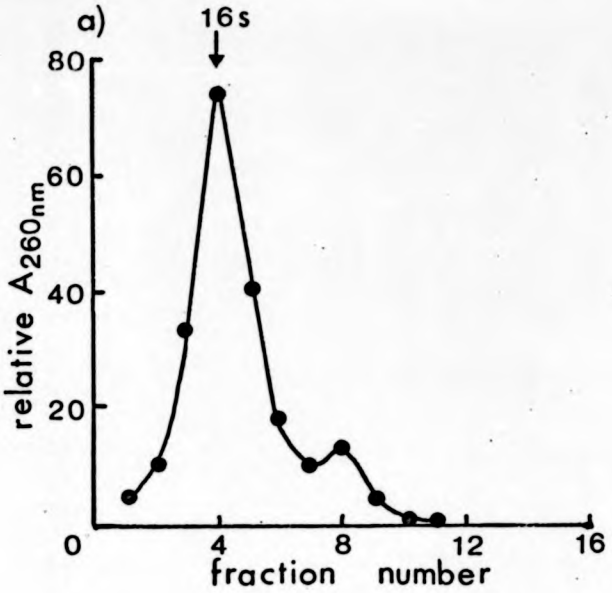
Fig. 7.6. A_{260nm} profile of purified 16s rRNA and 23s rRNA and 23s rRNA fractionated on sucrose gradients

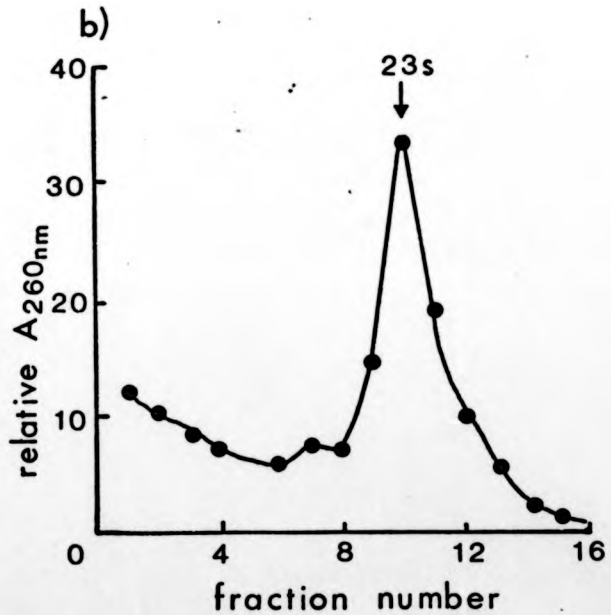
Sedimentation was from left to right. Units of absorbance were relative to a base line of 0.2 units/ml.

Similar fractions from several gradients like the one shown in Fig. 7.5. were pooled, concentrated, and then purified by further fractionation on 5-20% sucrose gradients. Peak fractions were again pooled and concentrated.

a) Purification of 16s rRNA

b) Purification of 23s rRNA


a)
16s
80
60
40
20
0
relative A_{260nm}
4
8
12
16
fraction number


b)
23s
40
30
20
10
0
relative A_{260nm}
4
8
12
16
fraction number

It can be seen that the 23s rRNA peak was more contaminated with 16s rRNA than vice versa. Peak fractions were again pooled and reprecipitated with ethanol.

Both ribosomal RNAs were labelled at their 5' ends with the aid of the enzyme T1 polynucleotide kinase and γ^{32}P-ATP (2.30). After repeated phenol extraction and ethanol precipitation to remove unbound ATP, the probes had a specific activity of 10^5 cpm μl^{-1}, which was suitable for hybridization.

7.3.3. Transfer of DNA fragments from agarose gels to nitrocellulose filters. R.vannielii DNA Fco RI-restriction fragments in an agarose gel were transferred to nitrocellulose by the method of Southern (1975). Minor modifications made to the procedure are described in 2.29.6. The filters were baked at 80°C for 2 hours to ensure that the DNA was firmly bound to the nitrocellulose. Over-baking of filters leads to their becoming very brittle and breaking up on further handling. Baked filters may be stored indefinitely before use.

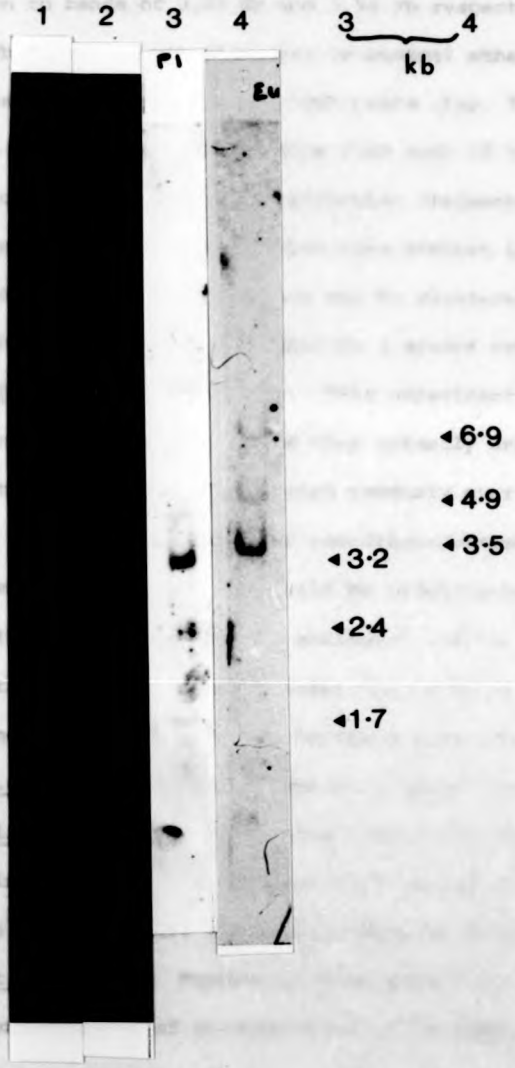
7.3.4. Hybridization of radioactive rRNA to Fco RI-restriction fragments of DNA. After pre-hybridization of the filter with yeast RNA, both rRNA probes were hybridized separately to Fco RI restriction fragments of R.vannielii DNA (2.31.1). Hybridization occurred overnight at 70°C, and after thorough washing and RNase treatment, the filters were air-dried and autoradiographed. The autoradiographs and corresponding gel are shown in Fig. 7.7.

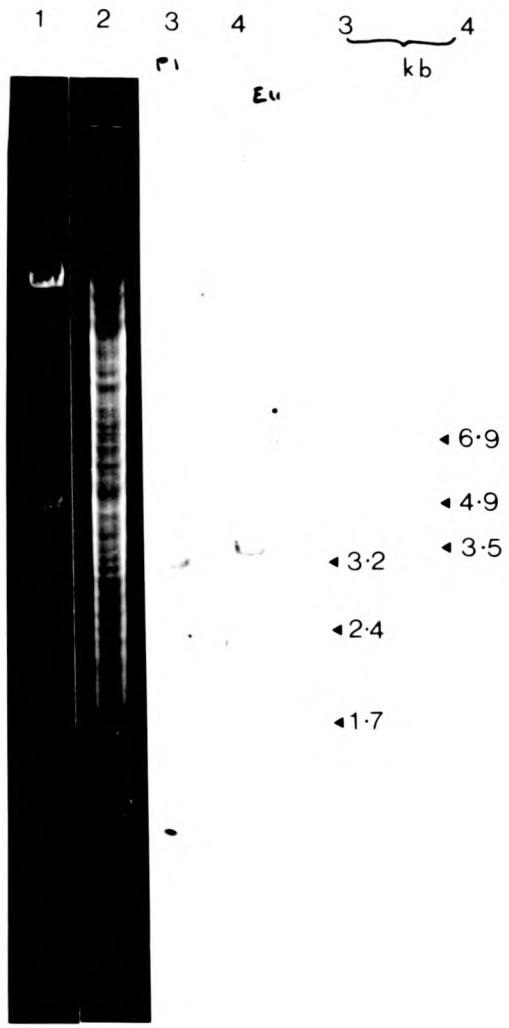
The autoradiographs show discrete bands of radioactivity indicating hybridization of RNA to specific DNA fragments. The molecular weights of these fragments are shown in the figure. The hybridization pattern implies that the rRNA genes are not randomly

Fig. 7.7. Location of DNA restriction fragments containing 16s and 23s rRNA genes of R.vannielii

EcoRl-restricted R.vannielii DNA was electrophoresed in 0.6% (w/v) agarose, and then transferred to nitrocellulose (2.29.6; Southern, 1975). Radioactive 16s and 23s rRNA probes were hybridized to the filter overnight at 70°C (2.31.1), and the washed filter was autoradiographed for 4 days at -70°C.

1. λ DNA restricted with EcoRl and HindIII
2. R.vannielii DNA restricted with EcoRl
3. Autoradiograph of the hybridization of a 16s rRNA probe to EcoRl-restricted R.vannielii DNA
4. Autoradiograph of the hybridization of a 23s rRNA probe to EcoRl restricted R.vannielii DNA.


1
2
3
4
3
4
kb
Pl
Eu
6·9
4·9
3·5
3·2
2·4
1·7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4
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kb
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4·9
3·5
3·2
2·4
1·7

distributed over the entire genome, but are confined to certain regions. Both the 16s and 23s rRNA probes showed a most intense hybridization to bands of 3.23 Kb and 3.54 Kb respectively. A lower amount of hybridization was also seen to several other distinct fragments, especially with the 23s rRNA probe (Fig. 7.7).

7.3.5. Conclusions. The finding that each of the rRNA probes hybridized to several unique DNA restriction fragments indicated that multiple copies of the rRNA genes were present in R.vannielii DNA. The 16s rRNA and 23s rRNA genes may be clustered on the chromosome, but they must be separated by a spacer region in which there is an Eco R1 restriction site. This experiment gives no information as to how close together they actually are. The evidence suggests that they were not distributed randomly over the entire chromosome. In view of the need for coordinated transcription of the genes, it seems unlikely that they will be widely separated in the genome. Further restriction enzyme analysis, and the use of double enzyme digestions are necessary to order the genes on the chromosome.

The genes coding for rRNA may be identified and amplified by cloning in E.coli. This would be done by ligating restriction fragments of R.vannielii DNA to a plasmid cloning vector and transforming E.coli. Clones carrying the rRNA gene sequence would be selected by colony hybridization to radioactive rRNA probes made as described above (Kennerley et al., 1977). Further studies would then be directed towards the organisation of transcription of the rRNA genes.

7.4. The origin of replication.

7.4.1. Introduction. As the origin of replication is an important marker, it would be useful to locate this on the R.vannielii chromosome. It is not possible at the present time to map the origin with respect to other genetic markers as in E.coli (Hiraga, 1976), but an alternative method developed for E.coli by Marsh and Worcel (1977) was adapted for use with R.vannielii. These workers were able to locate a specific 38 Kb DNA fragment which contained the origin of replication. Pulse-labelled DNA from a synchronous cell culture was digested with Eco R1, and the resulting fragments were separated on an agarose gel. After autoradiography the fragments into which label was first incorporated were visualized. A similar experiment was undertaken in R.vannielii, with a major modification being in the choice of radioisotope. As discussed earlier (4.2) (methyl-^{3}H)-thymidine is not incorporated into R.vannielii DNA and so alternative radioisotopes were used.

7.4.2. Attempts to locate the origin of replication. The following experimental approach to locate the origin was largely unsuccessful. However the experimental design is sound, and the experiments are described here, with possible reasons for their failure.

Since ^{3}H-adenosine was incorporated efficiently into DNA (4.2) it was used in the initial experiments. A homogeneous population of swarm cells was selected (2.12) and aliquots were pulse-labelled for 10 min. periods with ^{3}H-adenosine at intervals following the loss of flagellae from the swarm cells (2.27). This ensured that certain cells would be pulse-labelled at the onset of DNA synthesis. DNA was extracted from the cells, restricted with

Eco R1 and the resulting fragments were electrophoresed on a vertical 0.6% (w/v) agarose gel (2.29.2). DNA fragments were visible on the gel after staining, and the gel was fluorographed (Bonner and Laskey, 1974; 2.29.5), and then autoradiographed after pre-exposing the X-ray film to 1 ms light (Laskey and Mills, 1975). However, several autoradiographs were developed, and the whole experiment was repeated on three occasions, but no radioactivity was detected. Possible reasons for this were that there was insufficient radioactivity present, i.e. the pulse label time was too short to allow sufficient label to enter the pool, or the method of fluorography was unsuccessful.

Consequently, to avoid the use of fluorography, labelling the origin of replication was attempted with ^{32}P-orthophosphate. The experimental procedure was the same as described above except that the pulse label time was 15 min., and the swarm cells were concentrated by centrifugation immediately after selection, to increase the specific activity of phosphate in the exogenous phosphate pool. Autoradiography of the resulting gel produced only one clear band, but this was a high molecular weight fragment labelled with the last pulse, that is towards the end of DNA replication. This result is difficult to interpret, and was not repeatable. A problem which may arise is that the pulse label time was too short to allow equilibration of the exogenous and endogenous phosphate pools, and so incorporation of ^{32}P into DNA was low. It was not feasible to lower the exogenous phosphate concentration, since the swarm cells fail to differentiate in low phosphate concentrations, and consequently fail to replicate the DNA.

To circumvent the problem of the low specific activity of endogenous phosphate pools, a homogeneous swarm cell population was harvested by centrifugation, resuspended in phosphate-free medium, and

incubated in the dark for 1 hour with 2 μCi ml^{-1} ^{32}P-orthophosphate, to pre-label the endogenous phosphate pool. After this period the cells were transferred to the light and phosphate buffer containing a further 1 mCi ^{32}P-orthophosphate was added. The swarm cells developed normally, and samples were taken at regular intervals after the loss of flagellae. Subsequent manipulations were as described above and the autoradiograph showed that the DNA was successfully labelled, but no clear bands were distinguishable, and most fragments were labelled to some extent. In this experiment, since the radiolabel was present throughout, the pulse-labelling effect was lost. If many more samples were to be taken during the period when DNA replication was initiated, it may be possible to locate the first fragments to be labelled.

7.4.3. Conclusions. The results of this series of experiments are very inconclusive, and suggest that the experimental protocol needs further modification before meaningful data can be obtained. Marsh and Warcel (1977) had more success, perhaps because they were able to use greater numbers of synchronous cells, and the radioisotope they used radiolabelled DNA specifically,ensuring that DNA of high specific activity was extracted.

The number of swarm cellsused in the experiment could be increased, but this would necessitate using very much more isotope. The swarm cells may not be concentrated more than 10 fold, since they begin to show abnormal development. The use of ^{3}H-adenosine and an improved fluorography technique appear to offer the best prospects for further research in this area.

An alternative way of identifying the origin of replication would be to insert random R.vannielii DNA fragments into a vector

plasmid (e.g. pBR322 containing an auxotrophic marker) and use the hybrid plasmid to transform E.coli. Plasmids containing the origin should show a higher transformation efficiency, and in this way the fragment containing the replication origin may be located and analyzed further.

7.5. The nitrogen fixation genes.

7.5.1. Introduction. In the absence of fixed nitrogen, purple non-sulphur bacteria are able to fix atmospheric nitrogen. Nitrogen fixation is of great interest and importance to the world economy. The purple non-sulphur bacteria and the blue-green algae offer a unique opportunity for the study of both photosynthesis and nitrogen fixation in relatively simple organisms. Consequently both the biochemistry and genetics of nitrogen fixation have been studied intensively in recent years (Dalton and Mortenson, 1972; Stewart, 1973; Brill, 1975). Nitrogen fixation in R.vannielii was investigated for several reasons. At the start of this work difficulty was experienced in radioactively labelling DNA for kinetic experiments, and so the feasibility of density labelling the DNA with the heavy isotope of nitrogen (^{15}N) was explored. Analytical ultracentrifugation of labelled DNA (Meselson and Stahl, 1958) could be used to detect density changes in the sample, and hence indicate when DNA replication was initiated. The extent to which R.vannielii cells were able to fix atmospheric nitrogen in the presence of a small amount of fixed ^{15}N nitrogen was relevant to this study. The analytical work was halted when suitable radioisotopes for the labelling of DNA were identified (4.2).

The development of a genetic system for R.vannielii by the use of the plasmid R.68.45 (Chapter 6) facilitates the transfer and isolation of the nitrogen fixation (nif) genes, enabling molecular studies on their expression to be undertaken. The Gene Transfer Agent of R.capsulata (Marrs, 1974) was shown to mobilize nif genes accompanied by hydrogenase activity, indicating that the two functions are closely linked (Wall et al., 1975b), since the 'GTA'

can only mobilize short pieces of the chromosome.

If the level of nitrogenase activity is dependent upon nutrient conditions, then nitrogen fixation may be an inducible system, in which nitrogenase mRNA(s) are synthesized when required. If this is the case, the system will be useful in the investigation of transcriptional control and the regulation of mRNA synthesis.

Nitrogen fixation genes from Klebsiella pneumoniae have been cloned (Riedel et al., 1977) along with a his D^+ allele in a pMB9 vector, and used to transform E.coli. Since nif^+ clones were difficult to detect in recombinants, the his allele, adjacent to nif, was included to facilitate selection of nif^+ colonies. This procedure led to the demonstration that two competing controlling elements for nif transcription are operating in K.pneumoniae.

With the development of a genetic system for R.vannielii, similar experiments should be possible to examine the expression of the nif genes in the bacterium. The inducibility of the nitrogenase enzyme system was investigated with this aim in mind, and the preliminary data obtained are discussed.

7.5.2. Effect of fixed nitrogen (ammonia) concentration on nitrogen fixation. A series of experiments was designed to examine the effect on nitrogen fixation of reducing the ammonium chloride (NH_4Cl) concentration in the culture medium. Since ^{15}N in the form of $^{15}NH_4Cl$ is very expensive, it was necessary to keep the concentration of $^{15}NH_4Cl$ at the minimum possible without reducing the growth rate.

The growth rate and nitrogenase activity of cultures at different concentrations of NH_4Cl, and grown under either argon or nitrogen gas, were examined. Growth rates were measured over an 8-day period (Fig. 7.8). With the usual NH_4Cl concentration

Fig. 7.8. Effect of NH_4Cl concentration on the growth rate of R.vannielii

▲ 0 µg ml^{-1} NH_4Cl

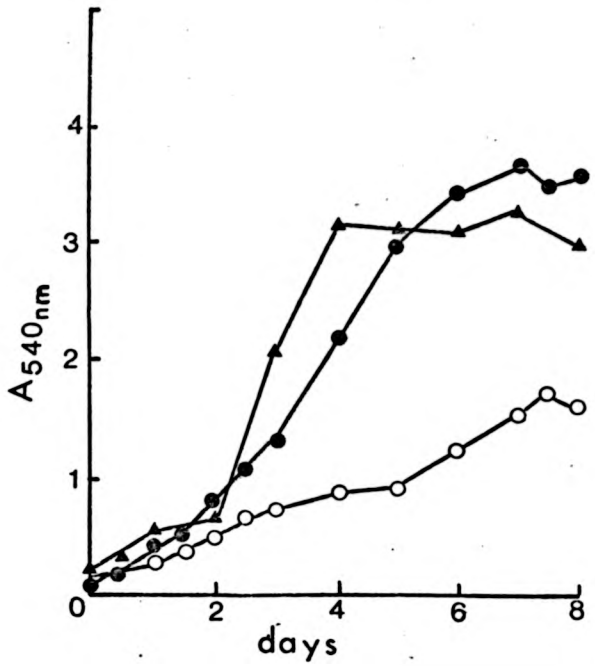
○ 50 " "

△ 200 " "

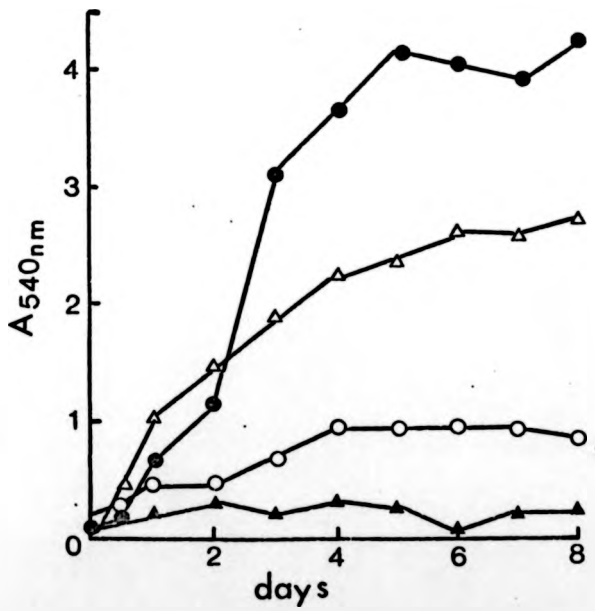
● 500 " "

R.vannielii (heterogeneous culture) was incubated with varying concentrations of NH_4Cl, and incubated phototrophically at 30°C either a) under nitrogen, or b) under argon. Growth (A_{540nm}) was measured with time (days).

a) under Nitrogen



b) under Argon



(500 μg ml^{-1}), growth was exponential until day 6 and then began to level off, whereas at lower concentrations growth was inhibited earlier, indicating that NH_4^+ ions were limiting. There was little significant growth in an argon-flushed culture with no fixed nitrogen, but the corresponding culture under nitrogen showed a growth rate which was almost indistinguishable from that of the 'normal' culture. Consequently in this culture the nitrogen fixation genes were fully induced.

The nitrogenase activity in each culture was measured at day 5, by subjecting a sub-culture to the acetylene reduction test (Bergersen, 1970; 2.18). Fig. 7.9. shows a comparison of the rates of acetylene reduction (i.e. nitrogenase activity) in each of the cultures. It was apparent that at low concentrations of fixed nitrogen, nitrogenase activity was increased. Nitrogenase activity was highest in the absence of ammonia and in the presence of nitrogen, but some nitrogenase activity was detected in the argon-flushed culture.

An absolute estimate of nitrogenase activity (Table 7.4) was obtained by relating the rate of acetylene reduction to the amount of protein per culture, as estimated by the method of Lowry et al. (1951) (2.16). Absolute nitrogenase activity was highest in the argon-flushed culture with no fixed nitrogen, since growth was most inhibited, and hence the cell number was low. Cells in this culture showed longer cellular filaments than usual, suggesting that stalk extension may be part of a mechanism for scavenging NH_4^+ ions. Nitrogenase activity was least in those cultures with 500 μg ml^{-1} fixed nitrogen, but it was not completely absent.

The activity of nitrogenase was dependent on NH_4^+ ion concentration (Fig. 7.10). When the NH_4^+ was reduced, nitrogenase activity increased exponentially.

Fig. 7.9. Effect of NH_4Cl concentration on the rate of ethylene production in the nitrogenase assay by R.vannielii.

▲ 0 µg ml^{-1} NH_4Cl

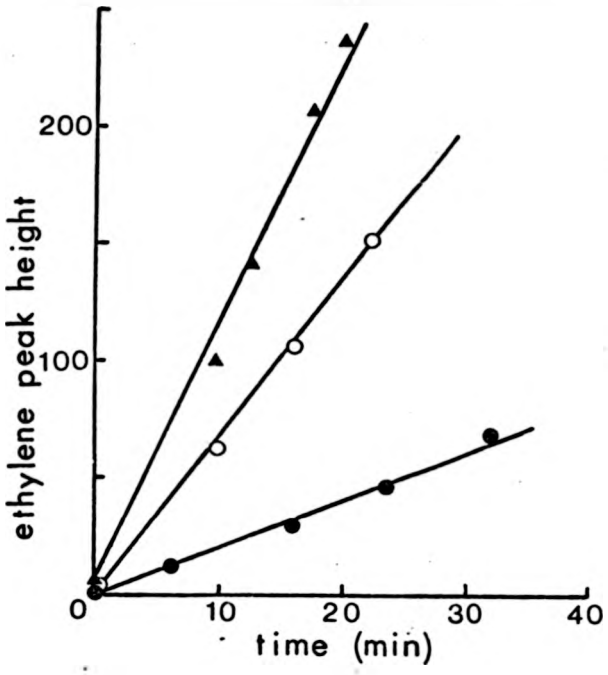
○ 50 " "

△ 200 " "

● 500 " "

Nitrogenase activity in R.vannielii incubated photoautotrophically at 30°C with varying concentrations of NH_4Cl and under a) nitrogen or b) argon, was measured by the acetylene reduction test (2.18) at day 5. Data were assessed by comparison of the rates of ethylene production, as measured by the increase in ethylene peak height.

a) under Nitrogen



b) under Argon

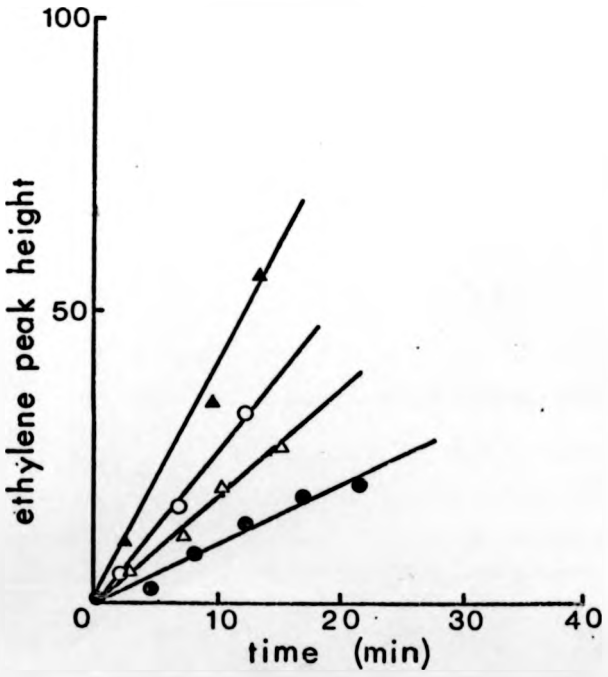


Table 7.4. Effect of NH_4Cl concentration on absolute nitrogenase activity in R.vannielii

	Concentration of NH_4Cl ($\mu g\ ml^{-1}$)	Rate of ethylene production per hr	Amount of ethylene in flask per hr	mg protein per flask	Nitrogenase activity (amount ethylene produced/hr/mg protein)
Under nitrogen	0	724	37.9	1.9	32.2
	50	384	20.1	0.62	32.4
	500	114	5.9	1.4	4.2
Under argon	0	156	11.9	0.14	84.7
	50	139	10.5	0.4	25.7
	100	164.1	15.8	0.8	19.2
	200	152.2	14.7	1.8	7.9
	500	50	3.8	3.3	1.14

Enzyme activity was assayed by the acetylene reduction method (2.18) and calculated as outlined in 2.18.

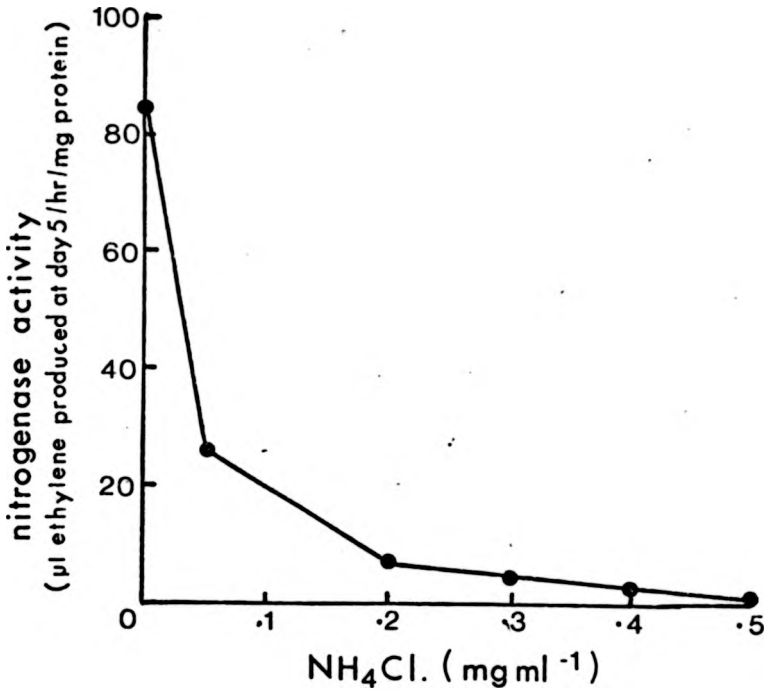


Fig. 7.10. Effect of NH_4Cl concentration on nitrogenase activity in R.vannielii

Absolute nitrogenase activity in R.vannielii under different NH_4^+ regimes was compared by relating the amount of ethylene produced in the acetylene reduction test to the protein concentration of each culture.

7.5.3. Conclusions and potential. Initial experiments on the regulation of nitrogen fixation in R.vannielii yielded promising results. A reduction in the concentration of NH_4Cl in the culture medium caused a reduction in the growth rate of R.vannielii cells, and an exponential increase in the activity of nitrogenase, although nitrogenase activity was not completely missing in cultures grown at the 'usual' concentration (500 µg ml^{-1} NH_4Cl).

Nitrogenase activity was not regulated by substrate concentration, since its activity was detectable in the absence of nitrogen, in the argon-flushed cultures. On the contrary, nitrogenase activity was regulated by ammonium ion concentration in the culture medium.

Increased nitrogenase activity in conditions where fixed nitrogen was low or absent, indicated that an inducible system was present. Consequently the nitrogenase enzyme(s) have great potential for studies of mRNA regulation and transcriptional control. The plasmid R.68·45 could be used to mobilize the nif genes from the wild type to complement nif$^-$ mutants of R.vannielii. This would facilitate isolation of the nif genes for further characterization and studies on their expression.

7.6. Conclusion.

Although the results presented in this chapter are preliminary and are far from complete, they serve to convey the idea that the recently developed hybridization and recombinant DNA techniques can be applied to understanding the regulatory mechanisms in organisms without a comprehensive biochemical and genetic background. These methods will be increasingly used in the study of morphogenesis and differentiation in both R.vannielii and other prokaryotic organisms. The future potential of these studies will be discussed in more detail in the final chapter.

CHAPTER 8

Conclusions and Potential

The aim of this thesis was to carry out a substantial investigation into the molecular biology of R.vannielii, in view of its potential for studies of microbial morphogenesis and differentiation. Particular attention was paid to the metabolism of DNA during the life cycle of the organism. The work described falls into two distinct categories. The first examines the control of cellular morphogenesis and differentiation at the molecular level in R.vannielii, and the second is an analysis of the genetics of the organism.

An initial study of the physical characteristics of R.vannielii DNA was made. The data described in Chapter 3 showed that DNA from all R.vannielii cellular expressions was similar in its melting and renaturation characteristics, and the lack of any significant difference in the frequency of repeated sequences implied that no permanent large scale gene amplification or other major alteration of the genome, occurred during cellular morphogenesis and differentiation. Short inverted repeat sequences, the only class of repeated DNA sequences found in R.vannielii, were common to all three cell types tested, so they are not implicated in any control mechanism. A recent report (Nisen et al., 1979) indicates that similar sequences in C.crescentus move around the genome during the cell cycle. In view of this it would be interesting to examine the distribution of the inverted repeats in the R.vannielii genome.

The major part of the work was centred on one particular part of the R.vannielii life cycle, namely swarm cell morphogenesis and differentiation. Differential gene expression must occur during this developmental sequence since different classes of protein appear and disappear during the period (Fig. 1.19). Regulation of gene

expression can occur at both the transcriptional and translational level, and studies are in progress to examine these processes in R.vannielii. The work in this thesis aimed to examine the inter-relationship between DNA replication and other recognizable events or 'landmarks' in the cell cycle.

Radiolabelling experiments and specific metabolic inhibitors were used to investigate the relationship between DNA replication and other biochemical and morphological events in the obligate differentiation of swarm cell to mother cell. Treatment of swarm cells with nalidixic acid indicated a 'dependent pathway' of cell cycle events, consisting of stalk synthesis, genome replication and cell division. This is apparent because cell division was absent, and stalk formation in the second cell cycle was much reduced when nalidixic acid was used to inhibit DNA replication. It may not be concluded that stalk synthesis is a prerequisite for DNA replication, since this has not been shown. However, this is probably the case since development of the stalk is necessary for the correct partitioning of the newly replicated genome into the daughter cell. DNA replication was a prerequisite for cell separation. Cell division itself was controlled by the daughter cell genome, as it did not occur in nalidixic acid-treated cells. This conclusion is supported by the finding that the 'plug' between cells always forms at a constant distance from the daughter cell, but its distance from the mother cell may vary.

Since a new daughter cell develops in the absence of DNA replication, the mother cell genome must remain transcriptionally active under nalidixic acid inhibition. In some cases the daughter cell is grossly distorted suggesting that under normal circumstances

the daughter cell genome exerts control over the pattern of protein synthesis. Since there is no data available about the half-lives of mRNA populations in R.vannielii cells, inferences concerning the origin of message for further cell development in nalidixic acid-treated cultures cannot be made.

The use of chloramphenicol as an inhibitor of protein synthesis enabled the relationship between protein synthesis and DNA replication to be examined. Labelling experiments (4.3) had shown that DNA replication in the uninhibited swarm cell was not initiated until near completion of stalk synthesis. There was a 'lag' or maturation period during which the flagellae were shed, and stalk formation began. Protein and RNA synthesis was detected during this period. The use of chloramphenicol demonstrated that the initiation of DNA replication had an absolute requirement for protein synthesis, whilst completion of ongoing rounds of replication continued in the absence of protein synthesis. This implies that the synthesis of specific initiation protein(s) is required prior to the onset of replication. Potential candidates for such proteins may be identified on SDS-polyacrylamide gels of proteins synthesized in cells at various stages in the differentiation sequence (Fig. 1.19). The putative 'initiation proteins' may be necessary for the formation of a replication complex on which faithful DNA replication can take place. There may also be a requirement for the synthesis of structural proteins involved in the stalk formation which is necessary prior to DNA replication.

The studies with chloramphenicol suggest a mechanism by which newly replicated DNA is transported into the daughter cell at the end of the stalk. One explanation of this process (Whittenbury and Dow, 1977), supposes that the growth point of the stalk and the point of

attachment of DNA to the membrane are one and the same, and that DNA replication occurs at the attachment point. Thus, as the stalk is synthesized, DNA replication occurs and one DNA molecule is 'dragged' into the stalk, and ultimately into the daughter cell. The other molecule remains in the mother cell (Fig. 8.1a).

However, the finding that DNA replication does not commence until some time after initiation of stalk synthesis suggests an alternative model. The DNA attachment site may be at the growth point of the stalk, but replication is delayed until after the stalk has reached a critical size, or another prerequisite biochemical event has been completed. As replication occurs, the daughter chromosome is segregated into the new cell, and the mother cell chromosome detaches from the membrane (Fig. 8.1.b). Reattachment of the genome at another site on the membrane is necessary prior to the development of a second stalk. Although DNA has never been visualized within the stalk, it may now be possible with electron microscope autoradiography. The purification of bacterial nucleoids (Evinger and Agabian, 1977) in which cells are gently lysed in order to isolate the chromosome along with associated cell membrane proteins, may indicate whether or not the chromosome remains attached to the cell membrane throughout the cell cycle. Another more exacting test of the two models described above would be to examine DNA replication in temperature-sensitive mutants, which at the restrictive temperature are able to make a stalk, but are unable to bud a daughter cell. Such mutants are not available at the present time.

In parallel with the biochemical studies, genetic analysis of R.vannielii was started, as it is only with an understanding of the genetics of the organism that a detailed analysis of the control of

Fig. 8.1. Models of daughter cell genome segregation in R.vannielii

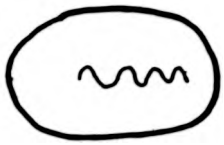
In model a) stalk formation and DNA replication are concurrent. Division of the growth point and replicon segregation occur simultaneously, the mother cell genome remaining in the stalk.

In model b) DNA replication commences towards the end of stalk formation. The DNA attachment point need not necessarily be at the growth point of the daughter cell.

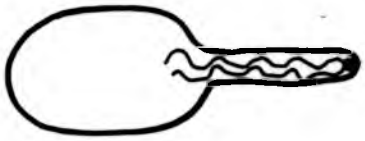
Upon replicon separation, the mother cell genome remains in the mother cell.

MODEL a).

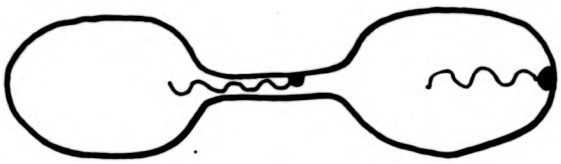
DNA free in
the cell



stalk formation and
DNA replication



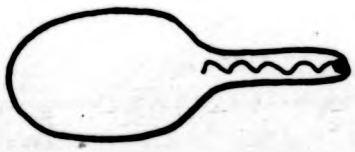
growth point division
and replicon segregation



MODEL b).

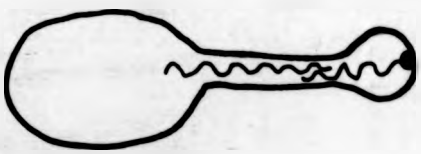
DNA free in
the cell

stalk formation



DNA replication

replicon separation
mother cell
genome detaches



differentiation can be undertaken. Prior to this investigation very little was known about the genetics of R.vannielii. Bacteriophages specific for R.vannielii had not been isolated, and neither conjugation nor the presence of pili had been demonstrated. Attempts to detect a native plasmid in R.vannielii were, surprisingly, unsuccessful in view of the ubiquity of plasmids among bacteria. The evidence seems to suggest that genetic exchange is a rare event in R.vannielii, yet the genome itself contains inverted repeat sequences which may act as foci for recombination, or points of excission and integration for transposons.

It is apparent from the data presented in Chapter 6 that genetic exchange can occur between R.vannielii and other bacteria by the demonstration of the transfer of the plasmid R.68.45 from E.coli to R.vannielii. Other members of the Rhodospirillaceae have been shown to transfer and maintain plasmids of the same incompatibility group. The use of R.68.45 offers a new and promising approach to the genetic analysis of R.vannielii, once stable auxotrophic mutants are obtained. Complex genetic systems such as those controlling morphogenesis and differentiation in R.vannielii should be amenable to study by using R.68.45 and related plasmids.

In addition to conjugation studies, the recently developed techniques of molecular genetics were applied to an analysis of the R.vannielii genome (Chapter 7). These methods have been used increasingly for the analysis of a wide range of problems in biology with dramatic success. Restriction enzyme cleavage of R.vannielii DNA allowed the reduction of a large genome to smaller, manageable pieces more amenable to study. The enzyme Bam Hl did not cleave the genome at all, suggesting that all the sites for the enzyme in the genome are

methylated, although methylation of the DNA has not been directly demonstrated. The reduction of the genome to smaller segments allows the identification of specific coding regions, exemplified by the location of the ribosomal RNA cistrons (7.3). Further analysis of the ribosomal RNA genes would involve the purification and cloning of those fragments which hybridized to the ribosomal RNA probe. Although the RNA cistrons may be located by hybridization of rRNA directly to the recombinant colonies, for other less well-characterized genes this is not the case. However, it should be possible in the future to isolate by methods similar to that described above, genes such as those involved in nitrogen fixation or photosynthesis (with the use of mutants) and, more interestingly, those genes involved in the control of morphogenesis and differentiation. The initial investigation into the physiology of nitrogen fixation in R.vannielii (7.5) was directed towards this end.

Attempts to locate the origin of replication on a specific restriction fragment were unsuccessful. Perhaps the specific activity of the labelled fragment was below the level of detection. Alternatively, synchronization of the initiation of replication itself may not have been sufficient to permit the preferential labelling of the origin. Since the E.coli replication origin has now been cloned, it may be possible to locate the R.vannielii origin by hybridization to the E.coli origin if the two origins show some homology.

Although this work has been concerned primarily with the obligate differentiation of the swarm cell to the mother cell, it is apparent that the rest of the R.vannielii life cycle has interesting possibilities and potential for the study of morphogenesis and differentiation. The regulatory mechanisms implicit in the nature of swarm cell production during exponential growth may also be amenable to investigation.

The mechanism of microcolony production introduces the phenomenon of ageing in a bacterial system since a mother cell produces a limited number of daughter cells before reproduction ceases. In this instance a transcriptional switch may be involved, leading to (perhaps) an irreversible 'turn-off' of the replication machinery. Alternatively, ageing mother cell DNA may have accumulated too many errors, or be altered in some way such as in the pattern of methylation, and consequently not function in further rounds of replication.

Many of the problems discussed above are not easy to approach by conventional means at the present time. Nevertheless, the recent advances in molecular biology now make possible direct experimental approaches to these problems, although new questions concerning morphogenesis and differentiation must be formulated. A major obstacle to the progress of research in this field is the absence of mutants of all kinds. A wide range of auxotrophic mutants is needed for the preparation of a genetic map. This may be obtained by using the conjugation system described in Chapter 6.

The isolation of temperature-sensitive, cell cycle and motility mutants is an essential next step in the elucidation of the control mechanisms in morphogenesis and differentiation. These would be of use in any future application of recombinant DNA technology in this area. Consequently, it is imperative that problems in the mutagenesis of R.vannielii be circumvented, so that many mutants may be isolated and characterized.

In summary, this work has illuminated the relationship between DNA replication and other biochemical and morphological events in the cell cycle, and progress has been made towards a genetic analysis of the organism. These approaches have been complementary in furthering the knowledge of this fascinating area of microbial morphogenesis and differentiation.

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The Genome of *Rhodomicrobium vannielii*, a Polymorphic Prosthecate Bacterium

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The base composition of *Rhodomicrobium vannielii* DNA was found to be 62·2% GC, and the genome size was $2{\cdot}1 \times 10^9$ daltons. There was no detectable difference between DNA from each of the three cell expressions examined. Reassociation kinetics indicated that no large group of repeated sequences was present, but that 5% of the genome was composed of extremely rapidly reassociating sequences. No plasmids were detected. Electron microscopic examination showed that *R. vannielii* DNA contained short inverted repeat sequences on average 400 base pairs long. The possible function of these sequences is discussed.

INTRODUCTION

Rhodomicrobium vannielii (strain RM5), a member of the *Rhodospirillaceae*, exhibits a complex polymorphic cell cycle (Whittenbury & Dow, 1977; Fig. 1). Exponential growth is characterized by ovoid cells linked by cellular filaments or prosthecae (Staley, 1968), which may be branched, giving rise to multicellular arrays and to peritrichously flagellated swarm cells. The latter undergo an obligate, well-defined differentiation sequence which leads ultimately to the generation of a multicellular array. There are two types of cell division in *R. vannielii*, one in which cells in the multicellular array are separated by plugs within the prosthecae, and the second in which swarm cells are formed from the prosthecal tips by division indistinguishable from binary fission.

Homogeneous swarm cell populations can be obtained quickly and in quantity by the glass wool column technique of Whittenbury & Dow (1977). Such populations undergo synchronous, obligate cellular morphogenesis and differentiation and provide an attractive model system for the study of the regulation of these processes. Homogeneous populations of multicellular arrays (cells remain attached, and cell division is by plug formation) can be obtained by permitting swarm cell populations to grow for three generations, at which stage no motile cells are present. When *R. vannielii* is cultured under high CO_2 tensions (Dow & France, 1980) a 'simplified' cell cycle expression is induced. This vegetative cell cycle is essentially one of constitutive swarm cell formation, i.e. multicellular arrays are not formed, and cell division is exclusively by binary fission.

The control of cell morphogenesis and differentiation in *R. vannielii* presents many interesting questions. This paper describes the structure and organization of the *R. vannielii* genome, as a basis for further study on the molecular biology of these processes.

METHODS

Organism and culture conditions. Rhodomicrobium vannielii strain RM5, isolated from freshwater by C. S. Dow, was used throughout. Liquid cultures were grown in pyruvate/malate (PM) medium (Whittenbury & Dow, 1977) and incubated anaerobically with shaking at 30 °C and an incident light intensity of

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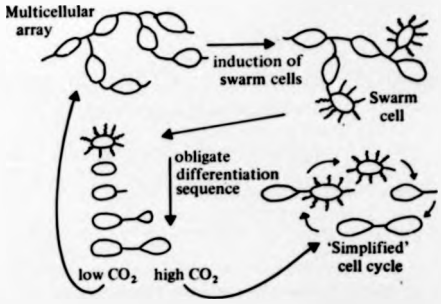

Fig. 1. The polymorphic cell cycle of *Rhodomicrobium vannielii*. There are three cell expressions: (*a*) multicellular arrays of 'mother' cells joined by prosthecae; (*b*) motile swarm cells; (*c*) 'simplified' cell cycle of swarm cells and stalked cells only arising under conditions of high CO_2 tension.

2000 lx. Cultures were incubated to the mid-exponential phase of growth (A_{440} about 2·5) prior to swarm cell selection.

Homogeneous populations of swarm cells were selected by filtration through a sterile glass column containing glass wool and beads (Whittenbury & Dow, 1977). These swarm cell populations showed 90% synchrony and had a generation time of about 6 h; 10^7 swarm cells ml^{-1} were routinely obtained.

Multicellular arrays, consisting predominantly of 'mother' cells, were obtained by allowing growth of homogeneous swarm cell populations for three generations. Cells showing the 'simplified' cell cycle were also used.

Preparation of DNA. All steps were carried out at 0 to 4 °C, unless otherwise stated. Cells were harvested by centrifugation at 5000 *g* for 15 min and washed in buffer (0·5 M-NaCl, 10 mM-EDTA, 0·1 M-Tris/HCl, pH 7·1). After resuspension in this buffer, lysozyme (Sigma) was added to a concentration of 500 $\mu g\ ml^{-1}$, and the cells were incubated at 37 °C for 1 h. Lysis was achieved by the addition of Sarkosyl (Koch Light; 1%, w/v) and incubation at 65 °C for 10 min. Pronase (Sigma; 50 $\mu g\ ml^{-1}$; previously autodigested for 2 h at 37 °C) was added and incubated overnight at room temperature.

Nucleic acids were purified from the cell lysate by two extractions with 1 vol. phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), saturated with SSC (0·15 M-NaCl, 0·015 M-trisodium citrate, pH, 7·0). After a final extraction with 1 vol. chloroform/isoamyl alcohol (24:1, v/v) the aqueous phase was precipitated with 2 vol. ethanol overnight at −20 °C. The precipitate was resuspended in 0·1 × SSC and digested with heat-treated RNAase (Sigma; 50 $\mu g\ ml^{-1}$) for 2 h at 37 °C. The DNA was then re-extracted as above.

Thermal denaturation. The DNA was further purified by hydroxyapatite chromatography (Britten *et al.*, 1974) and denatured by heat at a concentration of 100 $\mu g\ ml^{-1}$ in phosphate buffer (0·06 M-Na_2HPO_4/0·06 M-NaH_2PO_4). Hyperchromicity at 260 nm with increasing temperature was monitored in a Gilford recording spectrophotometer fitted with a reference compensator. The DNA base composition (mol % GC) was estimated from the measured T_m (i.e. the temperature at which half of the DNA was denatured), using the formula $T_m = 69{\cdot}3 + 0{\cdot}41$ (mol % GC), determined by Marmur & Doty (1962) for 0·2 M-Na^+. The effect of changing the Na^+ concentration from 0·2 to 0·18 M on the parameters of the formula for T_m is not significant (Marmur & Doty, 1962; our unpublished observations).

Reassociation kinetics. DNA (100 $\mu g\ ml^{-1}$) in phosphate buffer at 4 °C was sheared to a size of 1000 base pairs by sonication (5 × 1 min at 20 kHz and 1·5 A with a Dawe Soniprobe), prior to heat denaturation at 100 °C. This material was allowed to renature at ($T_m - 25$) °C, i.e. 68 °C, and the decrease in A_{260} with time was measured. The kinetics of the reaction were expressed by calculation of the C_0t (concentration × time, expressed as mol nucleotides l^{-1} s) required for half the reaction ($C_0t_{\frac{1}{2}}$; Britten & Kohne, 1968). Genome sizes were estimated relative to *Escherichia coli* K12 DNA ($2{\cdot}7 \times 10^9$ daltons; Klotz & Zimm, 1972) renatured under identical conditions, since in the absence of a large proportion of repeated sequences $C_0t_{\frac{1}{2}}$ is proportional to genome size.

Chemical estimation of the swarm cell DNA content. The DNA content of homogeneous swarm cells was determined by the modified diphenylamine method of Burton (1956), using calf thymus DNA (Sigma) as standard. Swarm cell numbers were determined on an electronic particle counter (Coulter Electronics, model ZBI).

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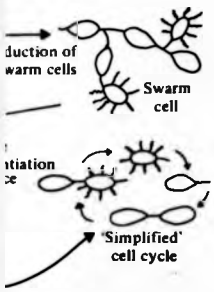

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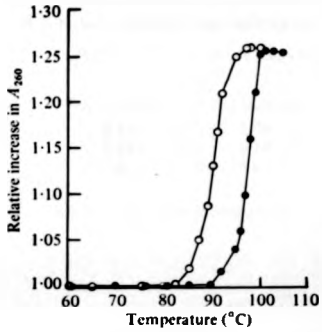


Fig. 2. Melting curves of *R. vannielii* DNA (●) and, for comparison, *E. coli* K12 DNA (○). DNA from the simplified cell cycle was isolated and thermally denatured as described in Methods.

Electron microscopy. Ethanol-precipitated DNA was resuspended in 1 mM-EDTA, 10 mM-Tris/HCl, pH 7·5. Part of each sample was sheared to a size of 20 kilobases by three passages through a 19 gauge needle, and both this and unsheared DNA were examined. The DNA was denatured by boiling for 10 min, then quenched on ice to prevent renaturation, and spread immediately by the aqueous technique of Davis *et al.* (1971). The concentration of DNA in the spreading solution was 1 μg ml^{-1} and the hypophase was 0·25 M-ammonium acetate, pH 7·5. Grids were stained with uranyl acetate (5×10^{-5} M), rotary shadowed with palladium/platinum wire, and examined in an AEI Corinth 275 electron microscope.

Size estimates were obtained by comparison with the double-stranded plasmid pBR322 ($2{\cdot}8 \times 10^6$ daltons; Sutcliffe, 1978: derivation, Bolivar *et al.*, 1977) spread under identical conditions.

Attempted plasmid isolation. [^{32}P]Orthophosphate (The Radiochemical Centre, Amersham) labelled cleared cell lysates (Clewell & Helinski, 1969) were banded in caesium chloride–ethidium bromide density gradients, as described by Radloff *et al.* (1967). The gradients were fractionated from the bottom and the fractions were precipitated with 10% (w/v) trichloroacetic acid on to Whatman glass fibre filters. The filters were washed, dried and counted in Triton/toluene scintillant in a Packard scintillation counter. The method of Currier & Nester (1976), for the isolation of large plasmids, was also used, involving selective chromosomal shearing and alkaline denaturation. Fractions from caesium chloride–ethidium bromide density gradients of preparations enriched for plasmids were collected and precipitated on to filters as described above.

RESULTS

This report describes the characteristics of DNA from *Rhodomicrobium vannielii*. In all the studies presented here DNA from swarm cells, multicellular arrays (predominantly 'mother' cells) and cells from the 'simplified' cell cycle were used.

Thermal denaturation

Figure 2 shows a melting profile of DNA from a simplified cell cycle culture, which has the characteristic shape of native double-stranded DNA. Melting curves of both swarm cell and mother cell DNA were identical to that shown. T_m values determined from the melting curves are shown in Table 1, along with the base compositions calculated from them. The average base composition value of 62·2 mol % GC from these denaturation studies compares well with the average value obtained for all three cell types by analytical centrifugation in a caesium chloride density gradient (62·5 mol % GC; Dow, 1972).

Table 1. *Characterization of DNA from Rhodomicrobium vannielii cell expressions and Escherichia coli* K12

	R. vannielii			
	Mother cell	Swarm cell	Simplified cell cycle	*E. coli*
T_m (°C)	97·2	95·0	97·0	89·5
mol % GC	61·8	62·3	62·7	50
$C_0t_{\frac{1}{2}}$ (mol s l^{-1})	4·5	4·7	4·3	6·4

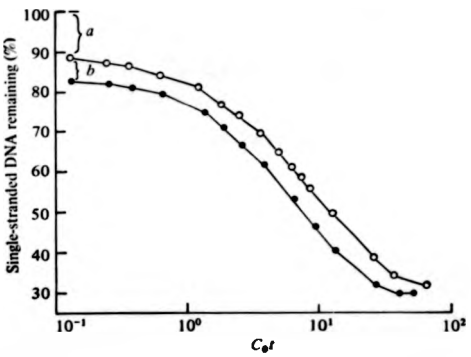


Fig. 3. Reassociation of *R. vannielii* DNA (●) and, for comparison, *E. coli* K12 DNA (○). DNA from mother cells was isolated, denatured and allowed to reassociate as described in Methods. The percentage of single-stranded DNA remaining was calculated from the hyperchromicity remaining at time t, expressed as a percentage of the total hyperchromicity. a represents the collapse hypochromicity and b the rapidly renaturing component.

Thermal reassociation

Figure 3 shows the reassociation kinetics of *R. vannielii* multicellular array (mother cell) DNA, compared with that of *E. coli* K12 DNA. DNA from both swarm cells and cells from the simplified cycle renatured in exactly the same manner. The shape of the curve indicates that *R. vannielii* contains no large proportion of repeated sequences in any of its cell types, and in this respect has a genome resembling other prokaryotes.

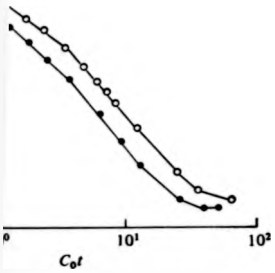
The $C_0t_{\frac{1}{2}}$ values determined from the reassociation curves of all three cell types are shown in Table 1, along with that determined for *E. coli*. An estimate of the genome size of *R. vannielii* may be obtained, as outlined in Methods, by comparing its $C_0t_{\frac{1}{2}}$ with that of *E. coli*; the genome sizes obtained in this way for the three *R. vannielii* cell expressions were the same (Table 2). The genome size of swarm cell DNA was also determined chemically by the diphenylamine reaction (Burton, 1956). Only swarm cell DNA was examined by this method, since a reliable cell count can only be obtained from a homogeneous swarm cell population. The chemical determination confirmed the value obtained by reassociation kinetics (Table 2). Thus *R. vannielii* has a genome size of about $2{\cdot}1 \times 10^9$ daltons, i.e. about two-thirds of the size of the *E. coli* genome.

The initial reduction in absorbance shown by *E. coli* DNA is termed collapse hypochromicity (Britten *et al.*, 1974) and is due to the restacking of bases on cooling. This well known phenomenon is shown by all DNAs when they are allowed to renature and accounts for approximately 10% of the hyperchromicity. In *R. vannielii* DNA there is an initial very rapid reduction in hyperchromicity of approximately 15%, i.e. more than can be

4 from Rhodomicrobium vannielii cell
Escherichia coli K12

R. vannielii

Swarm cell	Simplified cell cycle	*E. coli*
95·0	97·0	89·5
62·3	62·7	50
4·7	4·3	6·4



●) and, for comparison, *E. coli* K12 DNA (○). DNA
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Table 2. *Genome size of Rhodomicrobium vannielii cell expressions*

Values given (±standard errors) are the means of two to four determinations.

Cell expression	Genome size (daltons): Reassociation kinetics	Genome size (daltons): Chemical determination
Mother cell	$2{\cdot}10 \times 10^9 \pm 0{\cdot}2$	ND
Swarm cell	$2{\cdot}16 \times 10^9 \pm 0{\cdot}2$	$2{\cdot}13 \times 10^9 \pm 0{\cdot}3$
Simplified cell cycle	$1{\cdot}98 \times 10^9 \pm 0{\cdot}1$	ND

ND, Not determined (see text).

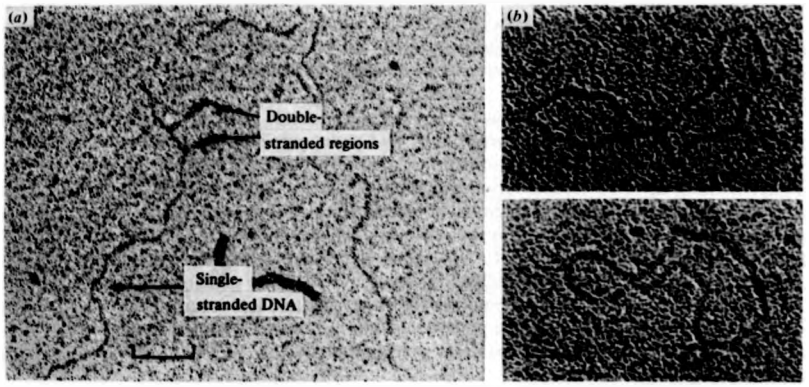

Fig. 4. Electron micrographs of (*a*) inverted repeat regions in *R. vannielii* DNA and (*b*) the plasmid pBR322 used for calibration. DNA was spread by the aqueous technique of Davis *et al.* (1971). Bar markers represent 0·1 μm.

ascribed to collapse hypochromicity. The 5% difference can be attributed to the presence of very rapidly reannealing DNA sequences of a kind not present in *E. coli* DNA. These sequences were found in DNA from all three cell expressions. The rate of reassociation of the sequences did not alter over a 10-fold DNA concentration range, suggesting that they were forming intramolecular hybrids.

Electron microscopy of DNA

Sheared DNA (from all three cell expressions) was denatured by boiling and then quenched immediately on ice to prevent renaturation. Figure 4 shows an example of the DNA molecules seen in the electron microscope and the pBR322 plasmid DNA used for calibration. Short regions of double-stranded DNA can be seen along the single-stranded *R. vannielii* DNA molecule. The size of these regions was estimated to be 400 base pairs long (±100 base pairs) by comparison with the plasmid ($2{\cdot}8 \times 10^6$ daltons) spread under identical conditions. Approximately 30 grids were examined.

DISCUSSION

The DNA base composition determined for *R. vannielii* is similar to those found for other members of the *Rhodospirillaceae* (Mandel *et al.*, 1971; Dow, 1972). Also, the kinetic

complexity measured for *R. vannielii* is comparable with that obtained for other bacteria. There appears to be no detectable difference between the genomes of the three cell expressions shown by *R. vannielii*, indicating that no large-scale gene amplification occurs during the differentiation process. An unusual feature of *R. vannielii* DNA is that about 5% of it is present as a rapidly renaturing component. This component is present in all three cell types.

The rapidly renaturing component could be a transient feature of replication, i.e. a 'knife and fork' structure (Barzilai & Thomas, 1970). However, since it was found in swarm cell DNA, where no DNA synthesis occurs (Whittenbury & Dow, 1977; L. E. Potts, unpublished results), and also in stationary phase cells, this possibility can be excluded.

An alternative explanation of the rapidly renaturing component is the presence of extrachromosomal DNA in the cell. However, we have been unable to detect plasmids in *R. vannielii* cells (results not shown; Dow, 1972), and there is no genetic evidence to suggest a plasmid is present. Similarly Wood *et al.* (1976) concluded that plasmids were absent from *Caulobacter crescentus*, a prosthecate bacterium, which also has an obligate dimorphic cell cycle.

The rapidly reassociating fraction formed hybrids at a rate independent of concentration, suggesting that intramolecular hybridization was occurring. This was confirmed by electron microscopic examination of heat-denatured, rapidly cooled DNA, which showed that adjacent short inverted repeats have reassociated, leaving the rest of the molecule single-stranded. Sequences similar to these have been found in both prokaryotic and eukaryotic genomes (Davidson *et al.*, 1973; Kopecko & Cohen, 1975; Schmid *et al.*, 1975), although their length and frequency varies. In *C. crescentus* they were found to occur approximately 300 times per genome and have an average length of 500 base pairs (Wood *et al.*, 1976). In *R. vannielii* they have an average length of 400 base pairs and it is assumed they represent the whole of the rapidly reassociating fraction of the genome, i.e. approximately 5%. Thus, they may occur approximately 200 times per genome, but at present nothing can be concluded about their distribution.

Inverted repeat sequences of about 130 base pairs in length have been implicated in recombination between plasmids pSC50 and pSC101 (Kopecko & Cohen, 1975). One can speculate that the inverted repeat sequences in bacteria may be 'hot spots' for recombination events.

Although the sequence complexity of *R. vannielii* is no greater than that of other bacteria, it does contain short inverted repeat sequences. The function of these sequences is unknown but they may be involved in recombination, or rearrangement of genetic material.

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