Biochemical and Molecular Genetic Approaches to Studying Protein Export by Cyanobacteria

by

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For my family

..... and to birding.

Olive-backed Pipit
Filey October 1987

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Declaration

I hereby declare that the work described in this thesis was conducted by myself, with the exception of those instances where the contribution of others has been specifically acknowledged. None of the information contained herein has been used in any previous application for a degree.

All sources of information have been specifically acknowledged by means of references.

D. J. Scanlan

David J. Scanlan.

Summary

We have shown that high molecular weight polypeptides are exported extracellularly by cyanobacteria. This is in addition to the well documented release of amino acids and peptides into the culture medium. *Synechococcus* R2 secretes two polypeptides of 14,400 molecular weight, and *Nostoc* sp. MAC a 43,000 molecular weight polypeptide. Their function is unclear, but we have some evidence that the 43,000 polypeptide may be involved in metal-binding.

Outer membrane proteins (OMP), i.e. carotenoid-containing cell wall fractions, were isolated from several cyanobacteria and a prochlorophyte, and these showed that the extracellular polypeptides were not just cell wall components released into the culture medium. Functional analysis of *Synechococcus* R2 OMP showed that these proteins were heat-modifiable, a characteristic of porins, and that the pattern of OMP could be modulated by sucrose, magnesium limitation, phosphate-limitation and iron limitation. The effects of iron limitation on the OMP pattern could be simulated by the addition of EDDA (0.004%) to normal growth medium. *Synechocystis* sp. PCC6308 also induced specific OMP under iron limitation. It is possible that *Synechococcus* R2 OMP are multifunctional. Antibodies raised against a *Synechococcus* R2 cell wall fraction cross-reacted with several other cyanobacterial OMP, and with an OMP fraction from *Prochlorothrix hollandica*. Using this antisera several positive plaques were obtained after screening a *Synechococcus* R2 λgt11 library. Antibodies against an iron-regulated inner membrane protein, and a carotenoid-associated thylakoid protein cross-reacted with OMP from *Synechococcus* R2, suggesting a family of carotenoid-containing membrane proteins may exist.

The *lacZ* gene, encoding the enzyme β-galactosidase, has been expressed in *Synechococcus* R2 on both a multicopy plasmid and when integrated into the cyanobacterial chromosome. The mechanism of integration relied on homologous recombination events between two pBR-plasmid derivatives. This required the construction of a universal recipient strain of *Synechococcus* R2.

A gene encoding the extracellular enzyme pectate lyase from an *Erwinia* sp. was introduced into the chromosome of *Synechococcus* R2, but no detectable enzyme activity was observed.

The transposon Tn*phoA* was introduced into a cyanobacterial shuttle vector, but was incapable of transposition. Similarly, transposon Tn5, although stably maintained in the *Synechococcus* R2 chromosome after its introduction via homologous recombination, was not capable of transposition when introduced on a suicide vector. Even though transposition of Tn5 was observed in another *Synechococcus* sp. no mutant phenotypes were observed. NTG mutagenesis of *Synechococcus* R2 and *Nostoc* sp. MAC produced various pigment mutants, but mutants incapable of growth in chelator-deficient medium could not be obtained. This may reflect the selection procedure.

The LacZ promoter probe has been constructed for use in *Synechococcus* R2 based on a multicopy plasmid capable of replicating in this organism. Differential expression of LacZ by *Synechococcus* R2 was observed under conditions of magnesium and iron limitation. Similar use of a kanamycin promoter probe suggests this construct will be useful for selecting strong promoter sequences via selection for high antibiotic resistance.

Chapter 1

Introduction

1.1 "The largest, most diverse and widely distributed group of prokaryotes"

The description of cyanobacteria that is the title of this chapter was written by Stanier and Cohen-Bazire (1977), and is now generally accepted as correct. These organisms perform an oxygenic "plant-like" photosynthesis, with a photosynthetic apparatus similar to that found in higher plants. In addition to producing chlorophyll a, cyanobacteria synthesize accessory light-harvesting pigments - phycobiliprotein complexes - which comprise varying proportions of phycoerythrin, phycocyanin and allophycocyanin. The blue, purple or red colouration of these pigments contribute to the organisms characteristic appearance - hence the traditional name of "blue-green algae". This term was inappropriate because of the largely bacterial nature of cyanobacteria, hence their current consideration as a major bacterial group (Bergey's Manual, in press). Genome complexity, transcription and translation are typical of other bacterial types. Genome sizes range from 3×10^6 base pairs (e.g. *Synechococcus* R2 PCC7942 and typical of most other bacterial genera) to 12×10^6 base pairs (Herdman *et al.*, 1979) which characterise the morphologically complex filamentous cyanobacteria (e.g. *Nostoc* sp. MAC PCC8009). RNA polymerases have an $\alpha_2 \beta\beta'\sigma$ structure and their activity is inhibited by rifampicin. The sedimentation value of ribosomes is 70S and translation is sensitive to typical prokaryotic inhibitors such as chloramphenicol and tetracycline. The cell envelope of cyanobacteria exhibits the characteristics of Gram-negative bacteria (see Drews and Weckesser, 1982) - but see Sections 1.3 and 4.1.

The great advances over the last decade in bacterial molecular biology has led to a concomitant upsurge in interest in the genetics and

molecular biology of cyanobacteria (for reviews see Doolittle, 1979; Tandeau de Marsac and Houmard, 1987; Shestakov and Reaston, 1987). Cyanobacteria in their own right are of great appeal to molecular biologists, most obviously for their oxygenic photosynthesis which allows a comprehensive genetic dissection. Bryant (1986) gives a detailed review of the cyanobacterial and higher plant photosynthetic apparatuses. However, their evolutionary position, cellular differentiation properties nitrogen fixation ability and ecological interest all require the application of modern molecular biological techniques.

Cyanobacteria are phylogenetically remote from the best studied bacteria, and more closely resemble the chloroplasts of eukaryotic algae - especially rhodophytes and cryptomonads (Bonen and Doolittle, 1976). This resemblance gave rise to the endosymbiont hypothesis, aptly summarised by Doolittle (1982) "protoeukaryotes first engulfed and later enslaved free-living oxygenic photosynthetic prokaryotes, of which modern plastids are the degenerate descendants". Chloroplasts exhibit a striking homology with contemporary cyanobacteria, and two cyanobacteria which have chlorophyll b and no phycobiliproteins as light-harvesting pigments have been isolated (Lewin, 1976; Burger-Wiersma *et al.*, 1986) However, we do not have any information as to whether either of these forms represent descendants of more immediate precursors to chloroplasts, or whether the alteration in light-harvesting pigments is an example of parallel evolution to that which has occurred in chloroplasts. Studies of cyanobacterial molecular biology then, have relevance in both prokaryotic and eukaryotic domains.

The photoautotrophic physiology of cyanobacteria provides a modal system for analysing gene expression in response to light, CO_2 or nitrogen availability, and contrasts that of the well-studied heterotrophic bacteria with a much wider and less focussed nutritional requirement. Processes of cyanobacterial photosynthesis and nitrogen fixation have succumbed to manipulation by molecular biologists in search of ways, as yet unsuccessfully, to increase photosynthetic efficiency or nitrogen-fixation capability.

Certain genera of cyanobacteria are capable of differentation of vegetative cells into heterocysts - specialised cells which maintain an anaerobic environment that facilitate the enzyme nitrogenase to fix atmospheric nitrogen (see Wolk, 1980). Heterocyst formation, as a strategy for nitrogen fixation is a feature unique to the cyanobacteria. The characteristic cellular spacing of heterocysts along a filament, together with the precise biochemical and genetic changes involved in their development, provides a model system for studying cellular differentiation (Adams and Carr, 1981). The formation from vegetative cells of spore-like resting cells or akinetes, which germinate under favourable conditions, hormogonia formation and the development of hair cells with the onset of phosphate deficiency are other important examples of cellular differentiation among the cyanobacteria.

Complementary chromatic adaptation - the ability of some cyanobacteria to modulate the relative abundance of their phycobiliproteins in response to light quality (Tandeau de Marsac, 1977), gas vacuole formation (Walsby, 1987), the widespread occurrence of cyanobacteria - from hot springs at temperatures over 70°C to under the ice of arctic

lakes, and the ability of some species to use hydrogen sulphide as electron donor (Padan and Cohen, 1982) suggests cyanobacteria will provide information on light regulation of gene expression, properties of enzyme stability and anoxygenic photosynthesis. At the genetic level, the application of the powerful techniques of bacterial genetics and recombinant DNA technology to cyanobacteria, will provide the basis for understanding such phenomena. The current state of cyanobacterial molecular biology is discussed in Section 1.8.

Recently, cyanobacteria have attracted interest in the biotechnology industry (Craig and Reichelt, 1986). As well as providing a nutritious form of protein (Klausner, 1986), certain cyanobacteria may be useful for producing ammonia on an industrial scale (Kirby *et al.*, 1986). However, an important and often forgotten property of cyanobacteria - one both unusual as well as open to dissection by current molecular biological techniques - is their capacity to export amino acids and peptides. The release of extracellular nitrogenous substances by cyanobacteria is particularly marked (see Sections 1.2 and 3.3.2) and this has obvious biotechnological application.

1.2 Cyanobacterial extracellular products

A great variety of extracellular substances are released by cyanobacteria (see Table 1.1), but although they appear to be widespread and to represent a significant proportion of productivity, we know very little about their composition and function. Fogg (1971) and Hellebust (1974) have reviewed the range of products excreted by algae including cyanobacteria.

Table 1.1 Summary of Cyanobacterial Extracellular Products

a) Extracellular Carbohydrates

Genera/Species	Carbohydrate	References
Anabaena, Nostoc	polysaccharides	see Fogg, 1971
Anabaena, Nostoc	simple sugars	Fogg, 1952
Calothrix, Scytonema (lichen symbionts)	esp. glucose & galactose	Chrost & Brzeska, 1978

b) Extracellular Organic Acids

Genera/Species	Organic Acids	References
Coccochloris, Anacystis	glycolate	see Fogg, 1971
Oscillatoria		

c) Extracellular Nitrogenous Substances

Genera/Species	Nitrogenous Substances	References
Anabaena, Calothrix	amides, amino acids	Fogg, 1952
Chlorogloea, Coccochloris	ammonia, peptides	Whitton, 1965,
Lyngbya, Microcystis	proteins	Walsby, 1974,
Nostoc, Oscillatoria		Antarikanonda, 1984,
Westiellopsis prolifica		

d) Extracellular Enzymes

Genera/Species	Enzymes	References
Coccochloris peniocytis	alkaline	Healey, 1973,
Anabaena variabilis	phosphatase	Doonan & Jensen, 1980
Anabaena, Nostoc	deoxyribonuclease	Wolk & Kraus, 1982

d) Extracellular Enzymes (/contd.)

Genera/Species	Enzymes	References
Coccochloris elabens	β-lactamase	Kushner & Breuil, 1977
Anabaena sp. PCC7120		
Anabaena	protease	Wolk, 1980

e) Extracellular Antimicrobial Compounds

Genera/Species	Antibiotic/Toxin	References
Scytonema hofmanni	cyanobacterin	Mason *et al.*, 1982
Anabaena flos-aquae	anatoxin A	
Anabaena, Nostoc	various	Flores & Wolk, 1986

f) Extracellular Polymers

Genera/Species	Polymer	References
Phormidium T-1	high molecular weight polymer	Fattom & Shilo, 1984

Substantial amounts of carbon compounds are released extracellularly, including glycollate produced as a result of photorespiration and polysaccharides, and these may provide an important energy source for bacteria in natural systems. Extracellular polysaccharides may also confer protection from desiccation and are probably involved in adherance to surfaces.

An extracellular high molecular weight polymer has been isolated from the benthic filamentous cyanobacterium *Phormidium* J-1 (Fattom and Shilo, 1984), which acts as an efficient bioflocculant and may play a role in clarification of the water column. This would provide a way of increasing the amount of ambient illumination at the benthic surface by reducing the turbidity of the surrounding water.

Cyanobacteria have been widely reported to secrete amino acids and peptides into their culture medium (Fogg, 1952; Antarikanonda, 1984). Other workers have shown that cyanobacteria indeed liberate substantial quantities of a great variety of extracellular nitrogenous compounds into the medium (Subramanian and Shanmugasundaram, 1986). *Anabaena cylindrica* releases about 30% of its organic nitrogen in rapidly growing cultures (Fogg, 1952), and similar relative amounts of extracellular nitrogenous material have been reported for other nitrogen-fixing cyanobacteria (14-42%).

It has been suggested that although amino acids and peptides are common in the supernatants of cyanobacterial cultures, they represent only a small part of the total extracellular material. A large proportion of the extracellular nitrogenous material is in the form of polypeptides,

and only small amounts of free amino acids are usually found (Fogg, 1952; Whitton, 1965).

Cyanobacteria release pigments, or substances that become pigmented, extracellularly. The brown pigment released by *Anabaena cylindrica* (Whitton, 1965) was partly formed from amino-acid containing UV-fluorescent compounds which became pigmented on polymerization, after their release into the culture medium (Walsby, 1974). The pigment-peptide just described complexes strongly any available iron from the culture medium, and this metal-ion-complexing activity may be an important function of such extracellular products. Copper, which complexes with the peptide bond, was also complexed by a peptide-containing fraction of a concentrated culture filtrate from *Anabaena cylindrica* (Fogg, 1952), and in this form was less toxic to the cyanobacterium. Whitton (1965) showed a decrease in the toxic effect of polmyxin B, a peptidic antibiotic produced by the bacterium *Bacillus polymyxa*, which inhabits the sheath of some cyanobacteria, by an extracellular polypeptide fraction produced by *Anabaena cylindrica*.

The production and excretion of antimicrobial compounds by cyanobacteria has also been reported. Cyanobacteria of many species produce toxins of peptide, phenolic and alkaloid nature. The hepatotoxins from *Microcystis aeruginosa* have been most studied and include a cyclic peptide of 1000 molecular weight, which contains novel amino acids (for review see Codd, 1984). However, there is no clear evidence that these toxins are exported, as distinct from being the products of cell lysis.

Mason *et al.* (1982) has described the production of a chlorine-containing antibiotic from the freshwater cyanobacterium *Scytonema hofmanni*. This antibiotic, named cyanobacterin, has been chemically characterised as a unique diaryl substituted γ-butyro lactone with a chlorine substituent on one of the aromatic rings. It specifically inhibits the growth of other cyanobacteria and a variety of eukaryotic algae but has no effect on eubacteria including the photosynthetic bacterium *Rhodospirillum rubrum*. Gleason and Paulson (1984) showed that cyanobacterin specifically inhibits photosynthetic electron transport.

The secretion of the mammalian neurotoxin, anatoxin A by *Anabaena flos-aquae* has also been described (see Codd, 1984). In addition, seven filamentous nitrogen-fixing strains of cyanobacteria were found to produce antibiotics very active against other cyanobacteria (Flores and Wolk, 1986). The antibiotic produced by *Nostoc* sp. 78-11A-E was a bacteriocin of low molecular weight, i.e. 'a proteinaceous antibiotic that is active against bacterial strains closely related to the bacterium producing the substance' (Flores and Wolk, 1986), whilst *Nostoc* sp. ATCC 29122 secreted, together with an antibiotic, a protein that inhibits its action.

Several enzyme activities have been detected extracellularly in cyanobacteria, including deoxyribonuclease (Wolk and Kraus, 1982), alkaline phosphatase (Healey, 1973) and protease (Wolk, 1980) activities. β-lactamase activity has also been found in a number of cyanobacterial strains including *Coccochloris elabens* PCC7003 and *Anabaena* sp. PCC7120 (Kushner and Breuil, 1977) but the authors were not certain whether the penicillinase(s) from these cyanobacteria could be

considered a "true" exoenzyme, one liberated from intact cells. This is an important criteria when defining an extracellular substance (see Section 1.3).

David and Thomas (1979) have suggested that extracellular polypeptides released by *Anabaena* L-31 under nitrogen-fixing and non-nitrogen-fixing growth conditions, functioned to either induce or inhibit heterocyst differentiation, but little further work has been done to confirm this potentially interesting observation.

Release of ammonia into the growth medium was initially reported to occur only when the activity of the ammonia-assimilating enzyme glutamine synthetase was inhibited by substances such as L-methionine-DL-sulphoxamine (MSX). This phenomena has been studied in different strains of cyanobacteria, fixing and non-fixing. More recently, however, certain strains of nitrogen-fixing *Anabaena* were found to release varying quantities of ammonia without any induction, both in the presence and absence of combined nitrogen (Subramanian and Sharmugasundaram, 1986). Immobilization and photoproduction of ammonia using these organisms, which is one of the emerging areas in cyanobacterial biotechnology, has since been reported (Kirby *et al.*, 1986).

The most common excretion products of *Anabaena* are polysaccharides and polypeptides. Among the polysaccharides, galactose and glucose are the dominant sugars (Chrost and Brzeska, 1978). Among excreted polypeptides and shorter peptides (including individual amino acids) serine was shown to be a significant constituent (Walsby, 1974). Bacteria attracted by

Anabaena showed strong chemotactic responses to many amino acids and sugars (Paerl, 1978). In particular, the amino acids serine showed consistent tendencies to attract these bacteria over a wide range of concentrations. Analyses of *Anabaena* and associated microorganisms have revealed the ability of bacteria to incorporate a range of amino acids and sugars. Other workers have accumulated evidence that extracellular products of cultured *Anabaena* are directly utilized by bacterial microflora associated with this bacterium (Chrost and Brzeska, 1978). Herbst and Overbeck (1978) demonstrated that the extracellular production of photosynthate by axenic cultures of *Oscillatoria redekei* could be utilized by associated bacteria. This nutrient exchange, between cyanobacteria and associated heterotrophic bacteria appears to confer no apparent selective advantage to the cyanobacterial partner. It is not clear however, whether cyanobacterial extracellular polypeptides are readily available in this way. Export of nitrogenous material by cyanobacteria in symbiotic relationships is well documented (see Meeks *et al.*, 1985).

1.3 Defining an extracellular product: selective membrane permeability and cell architecture

Fogg (1951) suggests an extracellular product denotes 'a soluble substance liberated from intact living cells and excluding soluble substances set free on injury or by the decomposition of dead cells'. Two types of extracellular product have been designated: type I include 'metabolic intermediates, usually of low molecular weight, for which there is an equilibrium between intra- and extracellular concentrations, so that the amount liberated varies according to metabolic activity'.

In cyanobacteria, these include organic acids, e.g. glycolate, and amino acids (Fogg, 1971). Type II: 'end-products of metabolism, usually of high molecular weight, the liberation of which does not depend on an equilibrium and which is more or less proportional to the amount of growth' include carbohydrates, peptides, enzymes, antibiotics and toxins. More modern definitions consider the relationship of extracellular products to membranes and membrane structure.

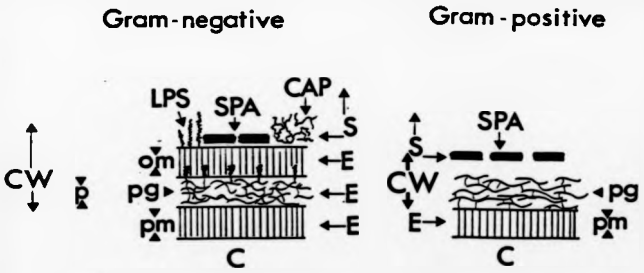
The ability of substances to be directed to the extracellular medium implies that they have traversed at least one membrane system. The selective permeability of the plasma membrane allows small molecules to diffuse into the cell down a concentration gradient. Plasma membranes are essentially lipid bilayers and although many substances can be transported across these, bacteria rely mostly on specific active transport systems for the accumulation of essential nutrients (Hengge and Boos, 1984). The vectoral arrangement of proteins in the membrane facilitates the movement of solutes across the bacterial plasma membrane bidirectionally. This allows the release of toxic biproducts of bacterial metabolism, extracellular antibiotic production and release of essential growth factors such as iron-sequestering siderophores, as well as energy-dependent transport systems for nutrient uptake.

Two different types of bacteria are distinguished by the presence (Gram-negative) or absence (Gram-positive) of a second membrane surrounding the plasma membrane. A schematic representation of these cell envelopes is described in Figure 1.1. All cyanobacteria so far studied possess an outer membrane, which indicates a Gram-negative nature, but on the other hand, the peptidoglycan layer is relatively thick and Jurgens and

Figure 1.1 Schematic representation of section through the cell envelope of a typical Gram-negative and a typical Gram-positive bacterium

C - cytoplasm; PM - plasma membrane; PG - peptidoglycan; P-periplasm; OM - outer membrane; CW - cell wall; LPS - polysaccharide chains of lipopolysaccharide; SPA - surface protein assay; CAP - capsule. Arrowheads indicate possible destinations for extracytoplasmic proteins. E - exported protein; S - secreted protein.

From Pugsley and Schwartz (1985).



Weckesser (1985) have suggested that a simple classification of the cyanobacterial cell wall into the Gram-negative type or the Gram-positive type might not be justified. This is considered in more detail in Chapter 4. Cyanobacterial cell envelopes typically possess an external layer - sheath or slime - bounding the outer membrane.

The bacterial plasma membrane contains a large number of protein species - up to 300 - involved in respiration, electron transport and nutrient uptake. The outer membrane contains considerably fewer proteins - perhaps 50 - involved in nutrient uptake, lipid metabolism and maintenance of cell wall integrity (for review see Hancock, 1987). Many outer membrane proteins serve as bacteriophage or colicin receptors (Pugsley, 1984). The periplasmic gel (Hobot *et al.*, 1984) between the inner and outer membranes contains a diffuse peptidoglycan linked to the outer membrane through the covalently bound lipoprotein. It contains approximately 100 proteins including binding proteins required for nutrient transport (reviewed by Ames, 1986) as well as hydrolytic enzymes such as phosphatases, nucleases, proteases and penicillinases. Additional surface layers (surface protein arrays, capsules) and surface appendages (fimbriae, pili and flagellae) may also be present in both Gram-negative and Gram-positive bacteria.

With reference particularly to extracellular protein production, the assignment of proteins to a particular compartment is defined according to cell fractionation criteria. Thus, high-speed centrifugation of disrupted bacteria separates membrane and soluble fractions. Inner and outer membranes can be separated on sucrose gradients, because they differ in density, as well as by their differential solubility in

detergents (Schnaitman, 1981). Periplasmic proteins may be released by osmotic shock (Neu and Heppel, 1965) or by chloroform (Ames *et al.*, 1984). We consider extracellular proteins to be those which are substantially and selectively enriched in cell free medium. Protein export defines protein translocation to an extracytoplasmic compartment of the cell (including the cytoplasmic membrane), and secretion to be the special case of protein export where the final destination is extracellular (Pugsley and Schwartz, 1985).

1.4 Extracellular proteins of bacteria

Three main classes of extracellular proteins of prokaryotes have been described. The first includes a variety of enzymes which degrade large polymers (nucleic acid, proteins, poly- and oligo-saccharides and lipids) to create smaller molecules which can be transported and metabolised by the bacterium. For example, α-amylase, β-glucanase and neutral protease secretion by various *Bacillus* species, pectinase, cellulase and protease secretion by *Erwinia* sp. and various degradative enzymes produced by *Myxococcus xanthus* (see Pugsley and Schwartz, 1985).

Bacteria also secrete a variety of toxins which are active against eukaryotic or prokaryotic cells, e.g. the heat-stable and heat-labile enterotoxins produced by *E. coli*, cholera toxin and α-haemolysin production by *Vibrio* sp., as well as lysins which degrade cell walls. Most bacteria are probably capable of producing and secreting bacteriocins, similar to the colicins produced by *E. coli* and related bacteria.

A third class of secreted proteins comprise enzymes which inactivate β-lactam antibiotics, including lipopanicillinases and β-lactamases produced by several members of the genus *Bacillus* (see Pugsley and Schwartz, 1985).

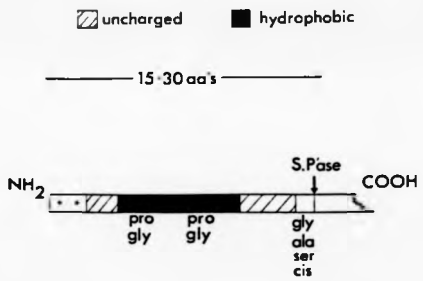
1.5 Mechanism of protein export

Numerous reviews on the export and secretion of proteins by bacteria are available (Michaelis and Beckwith, 1982; Pugsley and Schwartz, 1985; Holland *et al.*, 1986), and these reflect the current interest in this field. Therefore, only the salient features of this transport mechanism are described here.

The translocation of proteins into the periplasmic space of bacteria, is analagous to the initial step of secretion in higher organisms - the transfer of secretory proteins through the membrane into the lumen of the rough endoplasmic reticulum. In both systems the great majority of secreted proteins are initially synthesised with a largely hydrophobic N-terminal signal sequence, which is cleaved during translocation (Benson, 1985). A consensus structure of the N-terminal signal sequence is described in Figure 1.2. In eukaryotes, a signal recognition particle (SRP) binds the signal sequence and directs nascent polypeptides, together with associated ribosomal complexes to the membrane. Interaction with specific "docking" proteins - SRP receptor and signal-sequence receptor (Wiedmann *et al.*, 1987) then promotes translocation through the membrane. Cleavage of the signal sequence ensures translocation is complete and irreversible. Although a system corresponding to the SRP and docking protein components has not been

Figure 1.2 Consensus structure of the N-terminal signal sequence region necessary for membrane translocation

The extreme N-terminus usually carries one or more basic charges and the central core is composed of 11 or 12 strongly hydrophobic residues. Signal peptidase (S' Pase) located in the inner membrane of *E. coli* cleaves the signal sequence most frequently at a Gly or Ala residue. (From Holland *et al.*, 1986).



reported in bacteria, there is extensive genetical evidence that a similar machinery is found in *E. coli* (Gardel *et al.*, 1987).

A number of mammalian polypeptides carrying their own signal sequences are exported to the periplasm of *E. coli* with cleavage of the signal sequence at the normal site (Gray *et al.* 1985), and this indicates that overall mechanism of protein export is highly conserved between eukaryotes and prokaryotes.

Although a good general description of the export process is known, the type of amino acid sequences that prohibit transfer through the membrane is largely unknown. It is not enough to attach a signal sequence to a normally cytoplasmic protein and expect it to be exported (Pugsley and Cole, 1986; see also Section 7.1.3).

Two models have been proposed for secretion of proteins into the culture medium. In the first model, the secretion process occurs in one step (Lory *et al.*, 1983) - the protein moves from the cytoplasm to the culture medium through fusion zones between the inner and outer membranes - Bayer junctions (Bayer, 1979). The second model implies export to the periplasm, as described above, then secretion of the processed form of the protein across the outer membrane into the extracellular environment. Specific accessory molecules "exportases" may be involved, which interact with the secreted protein and allow its passage through the outer membrane (Pugsley and Schwartz, 1985). The specificity of such secretion mechanisms for a particular protein is not known, but the advent of transposon technology, e.g. the use of TnphoA (Manoil and Beckwith, 1985; Section 6) and the study of an amenable

system (Hinton and Salmond, 1987) should provide much information in this area.

1.6 Secretion of foreign proteins by bacteria

Gram-positive organisms such as *Bacillus subtilis* have become popular hosts for the synthesis and secretion of foreign proteins. These bacteria have a single surface membrane and normally secrete several enzymes at levels up to 10 g per l. For example, α-amylase is normally secreted at levels equivalent to 10-20% of total protein from industrial strains of *B. subtilis*. Engineering of such genes to produce a foreign protein fused to an N-terminal signal sequence and expressed from a suitable promoter has been reported (Vasantha and Thompson, 1986). However, a limited understanding of the genetics of these organisms, poorly characterized and often unstable plasmid vectors, and the release of proteases into the culture medium, which drastically reduces the yield of foreign proteins, has slowed progress in this area. The construction of strains lacking proteases will have great potential use for industrial secretion.

In Gram-negative bacteria such as *E. coli*, gene fusions have been used to promote the export of foreign proteins to the periplasm by coupling them to signal sequences. The use of *lky* mutants of *E. coli* (see also Section 3.1.2) may then have an important biotechnological role in differentially releasing proteins present in the periplasm. More interesting is the secretion directly into the culture medium of human β-endorphin using the *E. coli* OmpF protein signal sequence (Nagahari *et al.*, 1985). This underlines the importance of an analagous protein

export mechanism in prokaryotes and eukaryotes which cannot be understated when one is attempting to secrete foreign proteins in bacteria.

Cyanobacteria possess a number of advantages which might promote their use as hosts for secretion of foreign proteins. They are easily and cheaply maintained, and can be grown in bulk culture. Immobilization of cyanobacterial cells has been reported (Kirby *et al.*, 1986), and there have been numerous recent advances in their molecular biology (see Section 1.8). Elanskaya *et al.* (1985) has reported on the expression of the α-amylase gene of *Bacillus amyloliquefaciens* in *Synechococcus* R2, and hence vectors carrying the α-amylase gene and its regulatory elements may be useful for constructing plasmid molecules providing secretion of proteins from cyanobacterial cells. The exploitation of bacterial export systems in secreting foreign proteins has recently been reviewed (Nicaud *et al.*, 1986).

1.7 Nutrient transport and binding proteins

The existence in Gram-negative bacteria of periplasmically located substrate binding proteins which can be released from the periplasmic space by osmotic shock has been clearly demonstrated. A wide range of such proteins has been described, principally in *Escherichia coli*, that bind a range of compounds including sulphate, phosphate, galactose and a whole range of amino acids (see, for example, Rosen, 1978). The function of these proteins appears to be one of binding a substrate, the proteins possessing no demonstrable enzymatic activity. The physiological roles of such proteins appears to be 3-fold: they function in

transport systems, they are involved in chemotactic responses, and they are sensors of the environment with a potential role in metabolic regulation. No specific physiological information on such proteins in photo-autotrophic bacteria is available.

The importance of substrate transport systems under starvation conditions to *Escherichia coli* is such that they are constitutive. The advantages of such behaviour are obvious, in order to be competitive when nutrients become unvailable the cell must be able to assimilate such nutrients without delay. This is even more critical for bacteria growing in low nutrient environments.

Bacterial transport systems for many essential metabolites consist of an outer membrane receptor, a periplasmic shuttle protein, and a cytoplasmic membrane permease complex. Such a system has been developed by microorganisms for high-affinity iron assimilation (see Section 4.1.3). Much information on this process, mainly in the Enterobacteriaceae, has centred on the initial stages of this system, namely the binding of ferric-ion specific ligands (siderophores) to specific surface receptors and this is discussed more fully in Chapter 4. However, only recently in *Escherichia coli* has the involvement of a specific periplasmic protein in this high affinity iron uptake process been documented (Ozenberger, *et al* 1987).

1.8 Cyanobacterial molecular biology

Ever since the report of successful and reproducible transformation with the unicellular cyanobacterium *Anacystis nidulans* 602 (*Synechococcus*

PCC7943) (Shestakov and Khyen, 1970), there has been a steady increase in our knowledge of cyanobacterial molecular biology. Tandeau de Marsac and Houmard (1987) have recently reviewed these advances in cyanobacterial molecular genetics, whilst Shestakov and Reaston (1987) have reviewed gene-transfer and host-vector systems of cyanobacteria. Aspects of foreign gene expression, use of transposons and promoter probes in cyanobacteria are discussed in Chapters 5-7.

This thesis examines the process of protein export by cyanobacteria at several levels. Initially, the proteins exported by selected unicellular and filamentous strains were characterised both quantitatively and by SDS-PAGE. Rigorous criteria were determined to ensure that exported proteins were not a consequence of cell lysis. An analysis of the proteins exported to the outer membrane was then conducted and the characterisation of some of these proteins, in terms of their role in nutrient uptake, carried out. The later chapters have been directed towards a molecular genetic approach to studying protein secretion, specifically to characterise the export of foreign proteins introduced into cyanobacteria by appropriate vectors. Additionally, it was thought desirable to develop a system of transposon mutagenesis and to construct promoter probes for use in the study of this problem.

Chapter 2

Materials and Methods

2.1 Organisms

The cyanobacterial strain *Synechococcus* R2 (isolated by Grigoriava and Shestakov, 1976) was obtained from Professor G. van Arkel, Utrecht; this strain is physiologically similar to *Synechococcus* PCC6301 (*Anacystis nidulans*) but is distinct because of its transformation properties. *Nostoc* sp MAC was originally isolated from roots of the cycad *Macrozamia lucida* (Bowyer and Skerman, 1968), and now deposited in the Pasteur Collection as PCC8009. A complete list of cyanobacteria and of *Escherichia coli* strains is given in Table 2.1.

2.2 Plasmids and phage

Plasmids and phage used and constructed in this study are described in Table 2.2.

2.3 Chemicals

All media was made using analytical grade chemicals from BDH. Caesium chloride and acrylamide were purchased from Fisons plc, and N,N'-methylene bisacrylamide from the Eastman Kodak Co. Electrophoretic grade TEMED (N,N,N',N'-tetramethylethylene diamine), 2-mercaptoethanol and SDS-PAGE molecular weight markers were purchased from BioRad Laboratories, as was protein assay concentrate. Silver nitrate was obtained from Johnson Matthey Chemicals Ltd. Calcium chloride dihydrate, Grade I for transformation of *Escherichia coli*, Trizma base, the chromogenic substrates 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5-bromo-4-chloro-3-indolyl phosphate (XP) and methyl umbelliferyl-β-D-galactoside (MUG), methyl umbelliferyl phosphate

(MUF) were from the Sigma Chemical Company. Ethanol and methanol were reagent grade. Restriction enzymes and ^{55}Fe were from Amersham International, and ^{32}P from DuPont. Nitrocellulose filters were from Schleicher and Schuell.

2.4 Growth media

Cyanobacterial strains were maintained under photo-autotrophic growth conditions on the media listed in Table 2.3, described by Allen (1968) except that *Agmenellum quadruplicatum* PR-6 was grown in a synthetic sea water medium described by Rippka *et al* (1979) supplemented with vitamin B12 (10 $\mu g\ l^{-1}$). *Anabaena* CA was grown in Allen's medium modified by the addition of 4.39 g l^{-1} NaCl, 5.005 g l^{-1} $MgSO_4.7H_2O$. All media was made using analytical grade chemicals and purified water from a double ion-exchange unit plus carbon filter (Elgastat). Sterilisation was achieved by autoclaving at 15 lb sq. $inch^{-1}$ for 15 min. Four litre experimental cultures were grown in Allen's medium modified by the addition of $NaHCO_3$ 1.0g l^{-1}. Iron limited medium as specified in the text, contained Allen's medium as in Table 2.3, except that $FeSO_4.7H_2O$, 20 μM in normal medium, was reduced to 2 μM and 0.2 μM. Growth medium without added chelator contained no EDTA, $FeSO_4.7H_2O$ or citric acid. Iron replete medium plus added chelator contained Allen's medium and EDDA (0.004 % w/v). Media containing ammonia as the nitrogen source contained $(NH_4)_2\ SO_4$ at 2 mM and $NaNO_3$ was omitted. Sucrose, glucose and casamino acids were added as specified in the text after further sterilization.

Table 2.1 Organisms

a) Cyanobacteria

Strain	Source
Synechococcus R2	PCC7942
Synechococcus R2-SPc	PCC7942 (small plasmid cured)
Synechococcus sp.	Prof. N.G. Carr (this laboratory)
Synechocystis sp.	PCC6714
Synechocystis sp.	PCC6308
Synechocystis sp.	PCC6803
Agmenellum quadruplicatum PR-6	PCC7002
Anabaena variabilis	PCC7118
Anabaena CA	ATCC33047
Nostoc sp.	PCC6720
Nostoc sp. MAC	PCC8009

b) *Escherichia coli*

Strain	Phenotype	Source
DH1	F^-, recA1, endA1, gyrA96, thi-1, hsdr17 (r^-_k, m^+_k), supE44, relA17 λ^-	Maniatis *et al* (1982)
MC1061	araD139, Δ(ara,leu) 7697 ΔlacX74, galK$^-$, hsr$^-$, hsm$^+$, strA	Casadaban & Cohen (1980)
CC118	araD139, Δ(ara,leu) 7697, ΔlacX74, phoAΔ20, galE, galK, thi, rpsE, rpsE, $argE_{am}$, recA1	Manoil & Beckwith (1985)
S17.1	RP4-2-Tc :: Mu-Km :: Tn7 T^r, Sm^r, Km^s, Pro, res$^-$, mod$^+$	Simon *et al.* (1983)
Y1090	Δlac U169 proA$^+$ Δlon araD139 strA supF [trpC22 :: Tn10] (pMC9)	Young & Davis (1983)

Table 2.2 Plasmids, Phage

Plasmids

1) Cyanobacteria (*Synechococcus* R2)

Plasmid	Characteristics	Size	Source/Reference
a) Shuttle vectors			
pUC105	Ap^R, Cm^R	12.2	Kuhlemeier *et al.*,1981
pUC303	Sm^R, Cm^R	10.9	Kuhlemeier *et al.*,1983
pUC105XS	Ap^R, Cm^R, XhoI-SalI deletion	10.2	This study
pUC105H3	Cm^R, HindIII deletion pUC105	8.2	This study
pTUC1	Cm^R, Ap^R, lacZ	14.0	This study
b) Integrative vectors			
plAH4	Cm^R	7.7	S. Shestakov
plAH4 Sal	Cm^R, single HindIII site	5.7	This study
plAHPEL	Cm^R, pectate lyase gene	11.7	This study
plAH325	Ap^R, Cm^R contains pBR325	9.7	This study
c) Promoter probes			
pKIC-7	Ap^R, Km promoter probe		M. Alley
pLACPB1	Ap^R, Cm^R, lacZ promoter probe on shuttle vector	13.85	This study
d) Libraries			
pDAH221::	*Synechococcus* R2 chromosomal DNA		This study
pKIC-7 ::	*Synechococcus* R2 chromosomal DNA		This study
pLACPB1::	*Synechococcus* R2 chromosomal DNA		This study
pBR322 ::	*Synechococcus* R2 chromosomal DNA		This study

ii) *Escherichia coli*

Plasmid	Characteristics	Size	Source/Reference
pBR322	Ap^r, Tc^r	4.3	Balbas *et al.*,1986
pBR325	Ap^r, Cm^r, Tc^r	5.9	Balbas *et al.*,1986
pPEL	Ap^r, pBR322::pectate lyase gene	10.0	J. Hinton
pMAN007	OmpF[Fp-F], Ap^r	10.4	Matsuyama *et al.*,1984
pMAN006	OmpC, Ap^r	7.2	Matsuyama *et al.*,1984
pTBEG3	Ap^r, lacZ::pBR322	8.0	D. Hodgson
pDAH221	Km^r, Ap^r, promoterless lacZ, mob	11.2	D. Hodgson
pDAH216	Ap^r, promoterless lacZ	6.29	D. Hodgson
pHCP2	Ap^r, lamB gene	7.0	J-M. Clement *et al.*,1982
pSUP2021	pBR325::mob^+,::Tn5 Ap^r, Cm^r, Km^r		R. Simon *et al.*,1983
pRI46	Tn901, Ap^r		Kuhlemeier *et al.*,1981
RP4::Tn5	Tn5 carrying derivate of RP4-Km^s Ap^r, Tc^r, Km^r		J. Hinton
pRL1	ColE1 replicon, pDU1 replicon *bom* site, Cm^r		Wolk *et al.*,1984
pRL6	as above except Km^r		Wolk *et al.*,1984
pVWIC	Ap^r, Cm^r		Wolk *et al.*,1984
pJG28	Km^r, mob functions		Wolk *et al.*,1984
pDS4101	Ap^r, mob functions		Wolk *et al.*,1984

Phage

Phage	Characteristics	Source/Reference
λ::TnphoA	Transposon probe for exported proteins	Manoil & Beckwith,1985
λgt11	Expression vector	Young & Davis,1983

Table 2.3 Allen's medium

Salt	g l^{-1}	Microelements A6	g l^{-1}
$NaNO_3$	1.5	H_3BO_3	2.86
K_2HPO_4	0.039	$MnCl_2 4H_2O$	1.81
$MgSO_4 \cdot 7H_2O$	0.075	$ZnSO_4 \cdot 7H_2O$	0.222
Na_2CO_3	0.02	$NaMoO_4 \cdot 2H_2O$	0.391
$CaCl_2 \cdot 2H_2O$	0.034	$CuSO_4 \cdot 5H_2O$	0.079
EDTA	0.001	$Co(NO_3)_2 \cdot 6H_2O$	0.049
citric acid	0.012		
$FeSO_4 \cdot 7H_2O$	0.006		
microelements A6	1 ml l^{-1}		

2.5 Growth conditions and maintenance of organisms

Cyanobacterial cultures were grown at 34 ± 1°C in Allen's medium in an illuminated orbital shaker at a light intensity of 60-70 $\mu E\ m^{-2}\ s^{-1}$ (Vindon Scientific Ltd.) in a gas phase of 5% (v/v) CO_2 in air. For solid media, Allen's medium was supplemented with 1.5% (w/v) bacto agar and plates incubated at 30°C approximately 8-10 cm away from two warm-white strip lights (Osram 65/80 W). Double strength agar and growth medium were autoclaved separately and mixed at approximately 60°C. Cultures were maintained on 1.5% (w/v) agar slopes of Allen's medium in laboratory light avoiding direct sunlight. Contamination was checked by plating stock cultures onto nutrient agar and into nutrient broth, or onto Allen's medium supplemented with 10 mM glucose and 0.2% (w/v) yeast extract, and incubated at 30°C. Absence of bacterial or fungal growth after 7 days was taken to represent an axenic culture.

Table 2.4 *E. coli* growth media

	Constituents per litre	
Nutrient broth	13 g	Oxoid nutrient broth
Luria broth	10 g	Bactotryptone
	5 g	Bacto yeast extract
	5 g	NaCl [pH 7.2]
SOB medium (for high efficiency transformation)	20 g	Bactotryptone
	5 g	Bacto yeast extract
	10 ml	1 M NaCl (= 0.5844 g)
	2.5 ml	1 M KCl (= 0.186 g)
	10 ml	1 M $MgSO_4$, 1 M $MgCl_2$ filter sterile
Double Difco medium	20 g	Bactotryptone
	8 g	NaCl
	(+ 10 ml 20% (w/v) maltose, + 10 ml	
	1 M	$MgSO_4$ for λ::TnphoA work)
Pectinase assay medium	15 g	Bactoagar
	5 ml	20% (w/v) Bacto yeast extract
	5 ml	20% (w/v) $(NH_4)_2SO_4$
	9 ml	50% (v/v) glycerol
	125 ml	20% (w/v) polygalacturonic acid
	100 ml	phosphate buffer
Phosphate buffer	14.96 g	Na_2HPO_4 anhydrous
	0.34 g	$NaH_2PO_4.1H_2O$ (pH 8.0)

Escherichia coli was grown in liquid in nutrient broth or Luria broth (see Table 2.4) at 37°C in a Gallenkamp shaking incubator at 200 revs min^{-1}. Solid media contained in addition 1.5% (w/v) bacto agar. Nutrient agar (Oxoid) was also used. Liquid and solid media contained antibiotic where appropriate, at the concentrations described in Table 2.5. Cultures were stored at 4°C for 1-2 months, or in 15% (w/v) glycerol at -20°C for long-term storage.

Table 2.5 *Escherichia coli* antibiotic concentrations

Stock solution		Working concentration
Chloroamphenicol	24 mg/ml in ethanol	50 µg/ml
Kanamycin	25 mg/ml in water	50 µg/ml
Streptomycin	20 mg/ml in water	30 µg/ml
Ampicillin	25 mg/ml in water	50 µg/ml
Tetracycline	18.5 mg/ml solution in 50% v/v ethanol/water	12.5 µg/ml

2.6 Measurement of growth by dry weight estimation

A known volume of organisms was centrifuged at 10,000 rpm for 10 min, the pellet washed twice to remove salts in the medium, and aliquots of the suspension transferred to a preweighed vessel and dried to constant weight at 110°C for 20-24 hr. The extinction at OD_{750} of various dilutions of the organism suspension was measured, and a OD_{750} to dry weight relationship obtained.

Preparation of extracellular protein (see Chapter 3)

2.7 Quantitative analysis of extracellular protein

2.7.1 Ninhydrin method

This spectrophotometric method is based on the formation of a blue colour by reaction of ninhydrin and compounds having free amino groups including amino acids, peptides, proteins, primary amines and ammonia. The procedure used was based on the method described by Moore and Stein (1948). 0.1 ml of sample was placed in a 18 x 150 mm test tube and 1.0 ml of ninhydrin solution (Sigma Chemical Co.) was added. The tube was covered with an aluminium cap, vortexed, and heated for 20 min in a boiling water bath. 5 ml of diluent was added to each tube, the contents mixed, and readings taken on a LKB Ultrospec II spectrophotometer at 570 nm 15 min after removal of the tubes from the water bath. The standard curve was prepared with 0.1 ml samples of leucine at six concentrations from 0.5 to 2.0 mM.

2.7.2 Microbiuret method

This method, based on the ultraviolet absorption of the complex formed between protein and copper in strongly alkaline copper sulphate solutions, was performed as described by Itzhaki and Gill (1964).

2.7.3 BioRad protein assay

This method was described by Bradford (1976). A micro-assay procedure was routinely used. 0.8 ml of appropriately diluted samples were added to 0.2 ml dye reagent concentrate, gently mixed by inversion and the OD_{595} read against a reagent blank after twenty minutes. The standard curve was prepared with 0.8 ml samples of bovine serum albumin (BSA) at

five concentrations from 1 to 25 μg/ml.

2.7.4 Fluorescamine assay

This method has been derived from that described by Castell *et al.* (1979). It is based on the reaction of fluorescamine with primary amines yielding a fluorescent product. Proteins give normally higher specific fluorescence when reacted with fluorescamine than normal amines. However, the reaction with several purified proteins and polypeptides indicate that there is a considerable variation in the fluorescence of the different proteins when reacted with fluorescamine (Castell, *et al* 1979) and one must be aware of this. 20 μl of culture supernatant, or 0-40 μl of 0.25 mM leucine standard, were made up to 150 μl with water and 0.9 ml borate buffer added to each tube (0.2 M sodium borate buffer, pH 8.0 - 3.09 g boric acid per 250 ml H_2O, adjusted to pH 8.0 with 2 M NaOH). Whilst mixing the sample on a vortex mixer, 50 μl of fluorescamine reagent (fluorescamine (Sigma) 3.4 μg/ml in acetone dried over anhydrous $CaCl_2$) was added. A further volume of buffer was then added to each tube to bring the final volume to that appropriate for the fluorimeter; for the Perkin-Elmer 3000 this meant the addition of a further 1.0 ml. The fluorescence was measured as soon as possible using an excitation wavelength of 390 nm, and emission of 475 nm.

2.8 Qualitative analysis of extracellular protein

2.8.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of polypeptides was routinely performed using 10-30%w/v exponential gradient gels, on vertical Studier-type slab gel apparatus

(Hames and Rickwood, 1983). Gel solutions, made up to 100 ml with distilled water were prepared as follows:

a) 60% w/v acrylamide stock (high-bis): 60 g acrylamide, 1.6 g bis-acrylamide.

b) low-bis: 60 g acrylamide, 0.3 g bis-acrylamide

c) stacking gel acrylamide stock: 10 g acrylamide, 0.5 g bis-acrylamide.

d) Resolving gel buffer stock: 36.3 g Trizma base (3 M) adjusted to pH 8.8 with conc. HCl.

e) Stacking gelbuffer stock: 5.98 g Trizma base (0.05 M) adjusted to pH 6.8 with conc. HCl.

f) Reservoir buffer stock (10x conc.) per litre: 30 g Trizma base, 144 g glycine, 10 g sodium dodecyl sulphate.

The composition of 4% stacking gel and 10% and 30% resolving gel components is described in Table 2.6. Gels were run at 20 mA for 16 hr at 4°C.

Protein samples were denatured by addition of sample buffer and heated for five minutes in a boiling water bath, unless otherwise stated. The composition of sample buffer (4x conc.) was as described in Table 2.4. SDS-PAGE molecular weight markers (BioRad Laboratories) were run routinely on each gel. They comprised (molecular weight in brackets): phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400).

Table 2.6 Composition of 10-30% SDS-PAGE resolving gel, stacking gel, sample buffer

30% gel (20 ml)		10% gel (50 ml)	
10 ml	low-bis	8.3 ml	high-bis
7.3 ml	75% (v/v) glycerol	34.9 ml	water
2.5 ml	resolving gel buffer	6.25ml	resolving gel buffer
0.2 ml	10% (w/v) SDS	0.5 ml	10% (w/v) SDS
0.004 ml	TEMED	0.01 ml	TEMED
0.04 ml	10% (w/v) ammonium persulphate	0.1 ml	10% (w/v) ammonium persulphate

Composition of stacking gel		4x sample buffer (ml^{-1})	
3.0 ml	stacking gel acrylamide	0.25 ml	2x stacking gel buffer pH 6.8
4.4 ml	water	0.05 ml	glycerol (100% v/v)
2.4 ml	stacking gel buffer	0.4 ml	20% (w/v) SDS
0.1 ml	10% (w/v) SDS	0.2 ml	β-mercaptoethanol
0.005 ml	TEMED	0.1 ml	bromophenol blue
0.1 ml	10% (w/v) ammonium persulphate		

2.8.2 Staining of polyacrylamide gels

2.8.2.1 Coomassie blue staining

The staining solution contained 45% v/v methanol, 10% v/v glacial acetic acid and 0.1% w/v coomassie brilliant blue R250 (Biorad). Gels were stained for 2-3 hr and then destained in 45% v/v methanol, 10% v/v glacial acetic acid.

This procedure is insensitive, requiring about 100 μg total soluble cell protein per track (0.2-0.5 μg protein in a sharp band can be detected).

2.8.2.2 Silver staining

The method of Wray *et al.* (1981) was routinely used. This detects proteins at the nanogram level, is rapid, requires few reagents and little preparation and is reproducible. After electrophoresis the gel was soaked in 50% (v/v) methanol for at least 5-6 hr and then transferred to the stain solution for 15 min. The stain solution was freshly prepared by adding solution A dropwise to solution B with stirring, and made up to 400 ml with distilled water. (Solution A: 3.2 g silver nitrate in 16 ml distilled water; solution B: 84 ml 0.36% (w/v) sodium hydroxide + 5.6 ml 14.8 M ammonium hydroxide). After staining, the gel was washed twice in distilled water, before transferring to the developing solution (2.5 ml 1% (w/v) citric acid, 0.4 ml 38% formaldehyde solution made up to 500 ml with distilled water) for the required length of time.

2.8.3 Fluorography

Fluorographic detection of radioactivity in polyacrylamide gels with 2,5-diphenyloxazole (PPO), was performed as described by Hames and Rickwood (1983).

2.9 General molecular biological techniques

2.9.1 Restriction endonuclease digestion

The low, medium and high salt buffers were obtained from Amersham, at 10x concentration. After adding 1 μl of 10x restriction buffer for every 9 μl DNA, restriction enzyme was added and the digestion carried out at 37^{o}C for at least 1 hr. Spermidine (4 mM final concentration) was added to restriction digests of plasmid DNA prepared by the small-scale method.

2.9.2 Agarose gel electrophoresis

Horizontal agarose slab gels were prepared by boiling agarose (Sigma, Type 1 low EEO) in TBE buffer (0.089 M Tris-borate, 0.089 M Boric acid, 0.002 M EDTA) and 0.7% (w/v) gels were used routinely. The agarose was cooled to ca. 50^{o}C before pouring. DNA samples were prepared for loading by adding 0.2 volume of loading buffer (see Table 2.7). Electrophoresis was carried out with the gel completely submerged in electrophoresis buffer and run at 80 mA for 1 hr (mini gel system) or at 30 mA overnight. DNA was visualised by transillumination with short-wave UV light (260 nm) and photographed using Polaroid Type 665 film.

Table 2.7 Loading Buffer (gel beads) for DNA Agarose Gels

	per 10 ml
glycerol	2.0 ml
1 M Tris pH 8.0	0.1 ml
0.1 M EDTA	1.0 ml
H_2O	6.9 ml
agarose	20 mg
bromophenol blue	10 mg
xylene cyanol	10 mg
Orange G	10 mg

2.9.3 DNA restriction fragment isolation from agarose gels

Electroelution of DNA fragments from agarose gels was performed using an IBI electroelution chamber as recommended by the manufacturer.

2.9.4 Dephosphorylation of plasmid DNA

To restricted vector DNA, one-tenth volume 1 mM $ZnCl_2$, 10 mM $MgCl_2$, one-tenth volume 0.5 M glycine pH 9.4 (with NaOH), and 1-2 units of alkaline phosphatase (Boehringer Mannheim) was added, and incubated at 37°C for 30-60 min. The preparation was extracted with phenol:chloroform and the DNA ethanol precipitated (see section 2.9.7).

2.9.5 Ligation

Restricted DNAs and isolated DNA fragments were mixed in appropriate volumes of T/0.1E buffer (10 mM Tris, 0.25 mM EDTA pH 8.0). To subclone fragments, a 4:1 fragment:vector ratio was used, as described by Lagerski and Robberson (1985). Vector DNA was dephosphorylated as above, to prevent self-ligation. For library constructions, vector DNA was present in large excess. The ligation mixture was heated at 65°C for 10 min, and allowed to reanneal slowly on ice for 1 hr. After adding appropriate amounts of 10x ligation buffer (0.5 M Tris pH 7.4, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 10m M spermidine, 1 mg/ml BSA, 10 mM ATP) and T4 DNA ligase, the mixture was incubated at 15°C for at least 16 hr.

2.9.6 Extraction of DNA with phenol:chloroform

Phenol:chloroform was prepared by dissolving 100 g phenol and 100 mg 8-hydroxyquinoline in 100 ml chloroform and 4 ml isoamyl alcohol. This was equilibrated by shaking the mixture with two changes of 0.2 volume

1 M Tris (pH 8.0) and 2 changes of 0.2 volume TE buffer (10 mM Tris, 1 mM EDTA pH 8.0), before storage at 4°C. DNA samples were mixed with an equal volume of phenol/chloroform, until an emulsion formed. The two phases were separated by centrifugation in an Eppendorf centrifuge (4 min, high speed), and the upper aqueous phase removed. A further extraction with chloroform:isoamyl alcohol (24:1) was carried out as above, to remove any remaining phenol. DNA was recovered by ethanol precipitation.

2.9.7 Ethanol precipitation

To the DNA solution 0.1 volumes 3 M sodium acetate pH 5.6 and 2 volumes of ethanol (-20°C) were added, mixed by vortexing and chilled at -20°C overnight. The DNA was precipitated by centrifugation in an Eppendorf microfuge (15 min, high speed 4°C). The supernatant was discarded, the DNA dried under vacuum and the pellet resuspended in T/0.1E buffer.

2.10 Large-scale plasmid isolation from *Escherichia coli*

The procedure was based on that described by Clewell and Helinski (1970) for multicopy-plasmids.

An *Escherichia coli* culture (500 ml) grown in nutrient broth and appropriate antibiotic (and for pBR322-type vectors, amplified by addition of chloramphenicol to 300 $\mu g\ ml^{-1}$, OD_{600} culture = 0.4), was harvested by spinning at 8,000 rpm for 5 min at 4°C in a Hi-spin 21 centrifuge (MSE) using a 6 x 300 angle rotor. The supernatant was poured off, and the pellet resuspended in 16.5 ml Tris-sucrose (1.51 g Tris, 62.5 g sucrose pH 8.0, 250 ml). To the cells, transferred to SS34 Oakridge tubes (Nalgene Labware) 5 ml 10 $mg\ ml^{-1}$ lysozyme (Sigma) in

0.25 M Tris pH 8.0 was added, and the cells left on ice for 5 min. 4.5 ml 0.25 M EDTA pH 8.0 was then added, the cells incubated on ice for a further 5 min, before the addition of 18 ml lysis mix (500 ml lysis mix: 3.03 g Trizma base, 11.63 g EDTA, 10 g Brij 58, 2.0 g sodium deoxycholate pH 8.0). The tubes were inverted until cleared, which may require alternate incubation at 42°C. The cleared lysate was then spun for 15 min at 18,000 rpm, 4°C in a Hi-spin 21 centrifuge using a 8 x 50 angle rotor, which pellets unlysed cells, cell debris and chromosomal DNA, and 29 ml of this supernatant added to 29 g caesium chloride and allowed to dissolve. Ethidium bromide (3 ml 10 mg ml^{-1}) was added, and the solution loaded into a Beckman polyallomer heat seal tube. The preparation was spun for 16-18 hr in a VTi50 rotor (Beckman) in a Beckman L8 ultracentrifuge at 45,000 rpm, 15°C. The plasmid DNA, visualized using a hand-held longwave UV light, was removed through the side of the tube using a 21 gauge needle and 5 ml syringe and transferred to SS34 tubes. The DNA was extracted twice with water saturated butan-1-ol (which removes ethidium bromide), and to 3 ml DNA solution, 1.2 ml 1% sarcosyl, 1.2 ml 3 M sodium acetate pH 5.6, 6.6 ml T/0.1E and 24 ml 100% ethanol was added. Tubes were mixed by inversion and stored at -20°C overnight. The DNA was precipitated by centrifugation at 18,000 rpm for 40 min at 4°C in a Hi-spin 21 centrifuge using a 8 x 50 ml rotor. The supernatant was removed with a pasteur pipette using aspiration, and the pellet dried under vacuum. The pellet was resuspended in 0.5 ml T/0.1E, and the plasmid DNA stored at -20°C.

2.11 Small-scale plasmid isolation from *Escherichia coli*

The method usedwas a modification of the alkaline lysis method (Birnboim and Doly, 1979) as described by Maniatis *et al.* (1982).

Nutrient broth (5 ml) containing appropriate antibiotic was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking. Cells were harvested in an MSE Chilspin centrifuge at 5,000 rpm for 10 min, and resuspended in 150 µl of an ice-cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0. After transferring to 1.5 ml Eppendorf tubes, cells were stored 5 min on ice, and 200 µl freshly prepared lysis mix (0.2 M NaOH, 1% (w/v) SDS) was added. Tubes were inverted 2-3 times, stored on ice for a further 5 min, before the addition of 150 µl of an ice-cold solution of potassium acetate pH 4.8 (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml H_2O). After gentle vortexing, tubes were stored on ice for 5 min and spun for 5 min in an Eppendorf centrifuge. The supernatant was transferred to a fresh tube, an equal volume of phenol/chloroform added, mixed by vortexing and spun 4 min in an Eppendorf centrifuge. The supernatant was transferred to a fresh tube and ethanol precipitated (see section 2.9.7). The DNA pellet was resuspended in 30 µl T/0.1E pH 8.0, and 10 µl of this solution, subjected to restriction enzyme digestion. This required the addition of 1 µl of appropriate restriction enzyme buffer, 1 µl 40 mM spermidine, together with the desired restriction enzyme. The remainder of the DNA preparation was stored at -20°C.

2.12 Small scale plasmid isolation from cyanobacteria

Isolation of plasmid DNA on a preparative scale, followed the procedure of van den Hondel *et al.* (1979), as modified by Reaston *et al.* (1980). Allen's medium (100 ml) was inoculated with 0.5 ml of a well-grown culture and incubated for three days at 30°C. The cells were spun down in a Hi-spin 21 centrifuge (MSE) using a 6 x 300 ml rotor, washed once with 10 ml SE-solution (0.12 M NaCl, 0.05 M EDTA) and subsequently with 10 ml lysis buffer (25 mM Tris-HCl pH 8.0, 10 mM EDTA, 50 mM glucose). The cells were then resuspended in 0.5 ml lysis buffer and 0.25 ml lysozyme (10mg/ml, Sigma) was added. After 1 hr at 37°C the cells were lysed by the addition of 0.25 ml freshly prepared 10% (w/v) SDS. After 1 hr at 37°C, 0.25 ml 5M NaCl was added and after gentle mixing the lysed cells were left overnight at 4°C. The cell debris was removed by centrifugation for 30 min in an Eppendorf centrifuge at 4°C, and the supernatant was extracted twice with phenol and once with chloroform/isoamyl alcohol (24:1 v/v). The DNA was ethanol precipitated, vacuum dried and resuspended in 50 µl T/0.1E.

2.13 Chromosomal DNA isolation from cyanobacteria

Cyanobacterial chromosomal DNA extraction was based on a method described by Lind *et al.* (1985) with modifications.

A late-log phase culture (25 ml) was spun in a MSE multex centrifuge at 5,000 rpm for 10 min and resuspended in 0.5 ml 0.25 M Tris pH 8.0, 20% (w/v) sucrose containing lysozyme 10 mg ml^{-1}, and the cells incubated for 1 hr at 37°C. 16 µl 30% (v/v) sarkosyl and 20 µl 5 mg ml^{-1} proteinase K was then added, and the cells incubated at 65°C for 1 hr.

An equal volume of phenol:chloroform was added, mixed by vortexing and spun for 4 min in an Eppendorf centrifuge. The supernatant was dialysed overnight against TE buffer, and stored at -20°C. The DNA solution was concentrated by ethanol precipitation as required.

2.14 RNA isolation from cyanobacteria

The method described is as developed by M. R. K. Alley in this laboratory.

All solutions were made with sterilised water that had been previously treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC), a potent RNAse inhibitor, at 30°C overnight prior to autoclaving. This compound was also added (0.1% v/v) to all solutions, except those containing Tris, after their preparation and prior to their being autoclaved. Glassware was baked at 180°C for at least 3 hr.

A late exponential phase culture (25 ml) was centrifuged for 5 min in a MSE Multex centrifuge at room temperature in glass universals. The pellet was liquified and 1.8 ml 100 mM LiCl, 50 mM Tris, 30 mM EGTA, 1% (w/v) SDS pH 7.5 added. After 1-1.5 min at setting 10 in a Phillips Cooktronic 8915 Microwave oven, 1 ml acidic phenol (equilibrated with 50 mM sodium acetate pH 5.6) was added. The lysates were transferred to two Eppendorf tubes, and after vortexing at setting 9 (Vortex-Genie, Scientific Industries) for 30 sec, spun in an Eppendorf centrifuge for 2 min at room temperature. The top aqueous layer was removed and phenol extracted twice more, before ethanol precipitation. The RNA pellets were vacuum dried and resuspended in 400 µl DEPC treated water.

2.15 Southern blotting

After agarose gel electrophoresis the DNA was partially hydrolysed by acid purination - soaking the gel in 0.25 M HCl at room temperature until the bromophenol blue dye band turned yellow. Further denaturation of the DNA was achieved by soaking the gel in several volumes of 1.5 M NaCl, 0.5 M NaOH for 1 hr, before neutralising the gel by soaking in several voumes of a solution of 1 M Tris pH 7.4, 3 M NaCl for 1 hr at room temperature (Maniatis *et al.*, 1982). Transfer of DNA from agarose gels to nitrocellulose paper was as described by Southern (1975). After transfer was complete (usually after 12-20 hr) the filter was baked for 2 hr at 80°C under vacuum, before initiation of hybridisation experiments.

2.16 Northern blotting

A formaldehyde gel system was used as described by Maniatis *et al.* (1982), except that a different RNA loading buffer was used (see Table 2.8) which allowed the gel to be photographed before blotting.

Table 2.8 Loading Buffer for RNA Samples

		per ml
formamide (BRL)	(50% v/v)	500 µl
formaldehyde (BDH)	(16% v/v)	160 µl
MOPS 10 x		100 µl
glycerol	(10% v/v)	100 µl
10 mg/ml xylene cyanol	(0.025% w/v)	25 µl
10 mg/ml bromophenol blue	(0.025% w/v)	25 µl
10 mg/ml ethidium bromide		10 µl
water		80 µl

2.17 Radiolabelling DNA fragments

DNA was labelled to high specific activity using the technique described by Feinberg and Vogelstein (1984).

Plasmid DNA cleaved with appropriate restriction endonuclease was separated electrophoretically on a 1% (w/v) low gelling temperature agarose gel (BRL). The required band(s), viewed under UV light after ethidium bromide staining, were excised carefully with the minimum amount of extraneous agarose, and each band placed into a preweighed 1.5 ml Eppendorf tube. Water was added at a ratio of 1.5 ml H_2O g^{-1} agarose, and the tube placed in a boiling water bath for 7 min to melt the agarose and denature the DNA. Prior to initiating the labelling reaction the tube was kept at 37°C for 10-60 min. The DNA was stored at -20°C, and prior to subsequent labellings, the gel reboiled for 3 min and stored at 37°C as before.

The labelling reaction was carried out by addition of the following reagents in the stated order: 5 µl OLB buffer, 1 µl BSA, 16 µl DNA fragment, 2.5 µl ^{32}P α-dCTP (10 µCi/µl), and 0.5 µl Klenow large fragment DNA polymerase I (Amersham International), and incubated overnight at room temperature. The components of OLB buffer are described in Table 2.9.

Table 2.9 Composition of OLB Buffer

OLB is composed of the following solutions, A:B:C in a ratio of 10:25:15.

Solution O:	1.25 M Tris-HCl pH 8.0, 0.125 M $MgCl_2$. Stored at 4°C.
Solution A:	1 ml solution O + 18 µl 2-mercaptoethanol + 5 µl dATP, 5 µl dTTP, 5 µl dGTP (each triphosphate previously dissolved in 3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 at a concentration of 0.1 M). Stored at -20°C
Solution B:	2 M Hepes (titrated to pH 6.6 with 4 M NaOH). Stored at 4°C.
Solution C:	Pentadeoxyribonucleotides (Pharmacia) 50 OD units in 550 µl TE buffer to give a concentration of 90 OD units/ml.

Unincorporated nucleotide was removed by spinning the sample (made up to 100 µl with TE buffer) through a Sephadex G_{50} column at 2,500 rpm for 2 min in a Gallenkamp labspin centrifuge. The specific activity of the probe was greater than 10^7 cpm µg DNA^{-1}.

2.18 DNA-DNA and DNA-RNA hybridisation analysis

Pre-hybridisation and hybridisation of filters was carried out as described by Maniatis *et al.* (1982), except that the hybridisation solution contained 5 x SSPE (3 M NaCl, 0.2 M $NaH_2PO_4.2H_2O$, 20 mM EDTA, pH 7.4), 5 x Denhardts solution (5 g Ficoll 400, 5 g polyvinylpyrrolidone-10, 5 g BSA (Pentax Fraction V)/500 ml water - 50 x stock solution), 0.1% (w/v) SDS, ^{32}P-labelled denatured probe DNA and 100 µg/ml denatured salmon sperm DNA. The prehybridisation period was

4-5 hr, and the hybridisation period 12-16 hr.

For homologous probes filters were washed in 0.1 x SSPE, 0.1% (w/v) SDS for at least 2 hr at 65°C. Filters were exposed to X-ray film (Fuji RX) and stored at -70°C with an intensifier screen (Dupont, Cronex). The film was developed using Kodak LX-24 X-ray developer diluted 1+5 with water, and fixed with Kodak FX-40 X-ray liquid fixer, diluted 1+4 with water.

2.19 Transformation of *Escherichia coli*

Routine transformation with plasmid DNA was carried out as described by Maniatis *et al.* (1982), using calcium chloride grade I (Sigma). This method gave a transformation frequency of 5 x 10^5 transformants per μg pBR322 DNA for *E. coli* strain MC1061. For detection of β-galactosidase activity in transformants 50 μl of 2% X-gal and 50 μl of 100 mM IPTG was added in 4 ml 0.7% top agar.

For construction of genomic libraries an overnight culture of *E. coli* MC1061 was diluted 1:25 in 50 ml SOB medium (Table 2.4) in a 250 ml flask and incubated at 37°C, 275 rpm to OD_{550} = 0.35. Cells were then treated as described above. This modified procedure produced >10^6 transformants per μg pBR322 DNA.

2.20 Transformation of cyanobacteria

Transformation of *Synechococcus* R2 and *Synechococcus* R2-Spc (small-plasmid cured) was performed as described by Kuhlemeier *et al.* (1981).

A 2-3 day old culture grown with fast shaking was washed once in Allen's medium and resuspended to give a final concentration of 5 x 10^8 cells ml^{-1}. DNA (10 μl) was added to 0.2 ml cells in clear polypropylene tubes and incubated in the light for 30 min. Cells were plated on 1.5% Allen's agar under non-selective conditions and incubated for 16-18 hr in the light. Antibiotic (0.5 ml) was added underneath the agar at the desired concentration (see Table 2.10) and transformants detected after 5-6 days. Transformant colonies were restreaked on selective media. Modifications of this protocol are described in Chapter 5. For detection of β-galactosidase activity transformant plates were sprayed with a 10 mg/ml or 2 mg/ml solution of 4-methyl-β-D-umbelliferyl galactoside (MUG) in DMSO (Section 5.2.1) and the fluorescent product detected using UV illumination (Youngman *et al.*, 1985).

Table 2.10 Minimum inhibitory concentrations of antibiotic used for selection of transformants in *Synechococcus* R2 (-SPc)

Antibiotic	Concentration in solid/ liquid medium
Ampicillin	1 $\mu g\ ml^{-1}$
Chloramphenicol	7.5 $\mu g\ ml^{-1}$/5 $\mu g\ ml^{-1}$
Kanamycin	25 $\mu g\ ml^{-1}$
Streptomycin	10 $\mu g\ ml^{-1}$

Chapter 3

Cyanobacterial Protein Export:
Quantitative and Qualitative Analysis

3.1 Introduction

Bacteria that are able to secrete proteins into the growth medium occur relatively rarely amongst Gram-negative species. The fact that cyanobacteria do this, led us to examine the process of protein secretion in this group of organisms, whose mode of nutrition was fundamentally different from the heterotrophic bacteria. In spite of the apparent quantitative and biotechnological importance of cyanobacterial extracellular products, little is known regarding their composition, and less about the function of these extracellular proteins and peptides, some aspects of which will be discussed later.

We have screened several cyanobacterial strains quantitatively for the measurement of extracellular protein (see section 3.3.2), whilst a qualitative examination was limited to two strains, *Synechococcus* R2 PCC 7942, a transformable unicellular species, and *Nostoc* sp. MAC a filamentous species which we hoped to manipulate using a gene transfer system based on the conjugal shuttle vectors developed by Wolk *et al* (1984). In the long term, cyanobacteria might be considered as hosts for the secretion of foreign proteins, and this would require systems for cloning in, and genetic manipulation of, cyanobacteria. Later chapters will address these problems further.

We have considered extracellular proteins as those which are substantially and selectively enriched in cell-free medium, and for our purposes are those greater than 10,000 molecular weight.

Our main aims were:

i) To rigorously confirm whether or not cyanobacteria secrete proteins.

ii) Analyse the number, size and stage of production of such extracellular polypeptides.

iii) Assign a function to these secreted proteins, which would aid subsequent genetic manipulation, e.g. through a biological assay for screening gene libraries for a particular gene product.

iv) Ask whether such proteins have classical information, i.e. leader sequences, which allow their secretion.

v) Could the above information be used to investigate the secretion of foreign proteins in cyanobacteria?

3.1.2 Cell lysis, periplasmic leakage or true secretion?

With any study of extracellular protein release, one must distinguish between leakage and truely exported proteins, and for this we have employed specific criteria to help in establishing when cell lysis has occurred (Section 3.3.3). Such criteria have relied on the qualitative analysis of extracellular protein production by SDS-PAGE. However stringent one is to define true secretion though, one can still be open to criticism especially since the greatest release of extracellular material is often at the end of log phase when you might consider lysis to occur. Even more of a problem is the selective leakage of proteins from the periplasm. The use of *lky* mutants of *E. coli* in which proteins present in the periplasm are differentially released from the cells during growth, is one way however, of allowing normally periplasmic-located proteins to penetrate the outer membrane. This has important biotechnological implications.

3.2 Materials and Methods

3.2.1 Concentration of cell-free culture supernatants: means of recognising extracellular proteins

Preliminary experiments showed that a direct analysis of culture supernatants by SDS-PAGE did not show any extracellular protein bands even with silver staining, which can detect as low as 0.38 ng per mm^2 BSA. The culture supernatants were thus concentrated, using ammonium sulphate precipitation, concentration and ultrafiltration using the Amicon system, or lyophilization. The latter procedure was very time consuming, and produced a lyophilized supernatant with a very high salt content. As a result ammonium sulphate precipiation and Amicon ultrafiltration became the preferred methods.

To prepare an extracellular protein fraction, all work should be conducted with exponentially growing cultures, and it should be noted that the rate of secretion of proteins into the supernatant changes during exponential growth. Maximum rates were obtained at the end of exponential phase (see section 3.3.2). A procedure was optimized for obtaining extracellular proteins from both 100 ml and 4-litre batch cultures.

3.2.1a Small-scale 100 ml batch cultures

Organisms were centrifuged at 10,000 rpm in a Hi-Spin 21 centrifuge using a 6 x 300 angle rotor, for 10 min at 20°C (low temperature harvesting leads to lysis) and the supernatant passed through a 0.2 µm Millex sterilising filter. Total protein precipitation was obtained by addition of ammonium sulphate (47.2 g/100 ml yielding a 70% solution) which was ground to a fine powder and added to the supernatant at 4°C

over 10-15 min with stirring, and continued for a further 30 min before spinning in SS34 Oakridge tubes (Nalgene Labware) at 10,000 rpm for 40 min at 4°C. The supernatant was poured off, the tubes allowed to drain, and the pellet (which can be unstable and not always visible), resuspended in 0.5 M Tris buffer, pH 6.8. The sample was then subjected to SDS-PAGE.

3.2.1b Large-scale 4 L batch cultures

Organisms were centrifuged at 10,000 rpm for 10 min at 20°C as above, and protein in the supernatant was then fractionated by ultrafiltration through an Amicon hollow-fibre cartridge filter (type HIP10-8), giving a mean selection of 10,000 molecular weight and above. The supernatant was then passed through a 0.2 μm Millex filter and ammonium sulphate precipitated as above.

Euteneuer and Loos (1985) have remarked that concentration and ultrafiltration of culture supernatants by the Amicon system was very time consuming and a high loss of protein could be detected - the amount of protein being too low for gel application. In our hands, in tandem with ammonium sulphate precipitation, ultrafiltration using the Amicon hollow fibre small-scale system provided the only feasible means of concentrating large volumes of supernatant. It allowed simultaneous dialysis of the sample, but meant polypeptides smaller than 10,000 molecular weight (the size of the filter cut-off) were lost. Ultrafiltration of 4 L of supernatant can be completed in 3-4 hr - this is dependent on the specific hollow-fibre cartridge, and the flow rate used (see Table 3.1).

Table 3.1 Specifications of hollow fibre cartridge type HIP10-8 used in the concentration of cyanobacterial supernatants

Nominal cut off (molecular weight)	10,000
Surface area (m^2)	0.08
Internal fibre diameter (mm)	0.20
Cartridge diameter (cm)	2.30
Cratridge length (cm)	20.30
No. of fibres	1,000
Deionised water flow rate (ml/min)*	125-175
Recirculation rate (L/min)	0.2-0.6

* at 0.7 kg/cm^2 mean transmembrane pressure
Maximum operating pressure 1.1 kg/cm^2

From Amicon Publications.

3.2.2 Cellular fractionation procedures

Periplasmic fractions were obtained using i) the osmotic shock method based on Neu and Heppel, 1965, and ii) using chloroform (Ames *et al.*, 1984).

The osmotic shock procedure was as follows. To the bacterial culture, one-tenth volume 0.5 M Tris, pH 7.8 was added, and the cells incubated at room temperature for 10 min. Harvested cells were then resuspended in 800 µl sucrose solution (30 mM Tris pH 7.8, 40% (w/v) sucrose, 2 mM EDTA), transferred to 1.5 ml Eppendorf tubes and incubated for 10 min at

30°C. Cells were pelleted by spinning the tubes for 1 min in an Eppendorf centrifuge. The supernatant was removed very carefully, and the cells rapidly resuspended in 500 µl ice-cold distilled water, and incubated for 10 min on ice. Cells were pelleted by spinning the tubes for 1 min in an Eppendorf centrifuge, and the supernatant removed. This periplasmic fraction was stored at -20°C. The remaining cells were resuspended in 500 µl 50 mM Tris pH 7.8 and subject to two 15 sec bursts at amplitude 18 using a Jencons sonicator. Cells were kept on ice during sonication. The cell debris was removed by centrifugation and the remaining cytoplasmic fraction stored at -20°C.

Periplasmic proteins were also released using chloroform. Harvested cells (10 ml) were resuspended by brief vortexing in the residual medium, and 20 µl of chloroform was then added. After brief vortexing, the tubes were maintained at room temperature for 15 min, and then 0.2 ml of 0.01 M Tris-HCl pH 8.0 was added. The cells were separated by centrifugation for 1 min in an Eppendorf centrifuge, and the supernatant fraction containing the periplasmic proteins was withdrawn with a Pasteur pipette. This fraction was stored at -20°C.

3.2.3 Iron stain for iron-binding proteins in polyacrylamide gels

The method used was described by Chung (1985). It is based on the use of Ferene-S, a specific chromogenic ligand which gives a blue-coloured complex in polyacrylamide gels on interaction of the bound iron atoms of the protein.

After polyacrylamide gel electrophoresis the gel was immersed in 120 ml of staining solution, consisting of a mixture of 0.75 mM Ferene S and

15 mM thioglycolic acid (Sigma) in 2% (v/v) acetic acid, prepared just before use. This mixture was greenish-yellow. The gel background was cleared by destaining in 2% (v/v) acetic acid.

3.2.4 Assay of DNAase and RNAase activity

Supernatant from *Nostoc* sp. MAC or *Synechococcus* R2, removed at mid-late exponential phase of growth, was incubated at 37°C overnight with a solution containing 1 μg salmon sperm DNA or 1 μg calf-liver RNA, bovine serum albumin 100 μg/ml, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol. Control samples contained T/0.1E instead of cyanobacterial supernatant. DNAase or RNAase activity was assessed by agarose gel electrophoresis. Commercially available DNAase or RNAase was used as a positive control.

3.2.5 Assay of protease activity

Protease activity was assayed using i) protease substrate gel tablets (Biorad Laboratories) described in Biorad Bulletin 1111 (1982), and ii) using the chromogenic substrate azocasein (Braun and Schmitz, 1980) with concentrated and unconcentrated supernatants of *Nostoc* sp. MAC and *Synechococcus* R2. The reaction mixture contained 0.5 ml of azocasein (Sigma), 0.3 ml supernatant, 0.1 ml 1 M Tris-HCl pH 8.0, and 0.1 ml of distilled water. The reaction was stopped by the addition of 2 ml of 7% perchloric acid, and the mixture centrifuged for 3 min in an Eppendorf centrifuge. After addition of 0.3 ml of 10 N NaOH to 2 ml of the supernatant, the absorbance was determined at 436 nm. To the control samples, perchloric acid was added before the addition of azocasein. Appropriately diluted proteinase K was used as a positive control.

3.3 Results and Discussion

3.3.1 Suitability of various protein assay methods for quantiative measurement of cyanobacterial extracellular nitrogenous substances

Although there are many methods for estimating proteins, few are both sensitive and non-specific for type of protein (i.e. independent of composition). Thus, there are methods which measure peptide bond frequency, and those biased towards measuring the amount of a particular amino acid(s). To these considerations should be added the extent to which the method is open to influence by other chemicals, and its convenience.

Using the procedures for concentration of culture supernatants just described, quantitative estimation of extracellular protein became problematic, especially due to interference by salts, which limited the use of Biorad, Lowry and Biuret assays. Estimation of primary amino groups using a ninhydrin method, and a fluorescent assay (see section 2.7.4) with sensitivity in the nanogram range, became the preferred methods, and assayed culture supernatants directly for extracellular nitrogenous compounds without need of ultrafiltration techniques or ammonium sulphate precipitation. Ammonium sulphate interferes directly with these assay techniques.

The Biuret and microbiuret protein assays gave the lowest degree of variability between different polypeptides, as would be expected because they are specific methods for the peptide bond. However, a lack of sensitivity excluded their use from this analysis - requiring in excess of 100 μg of protein. Problems of chemical interference however, are

the main drawbacks of such protein assays, and careful consideration of this has to be given when choosing an assay technique.

The ninhydrin method was subject to much less interference, and though less specific for protein became a general assay for extracellular nitrogenous compounds. This lack of specificity is important when we consider that amino acids, peptides and ammonia may well be excreted by cyanobacteria.

For a measurement of the kinetic analysis of cyanobacterial protein secretion the fluorescamine assay was performed being very sensitive - requiring only 10-100 ng of protein - and allowing analysis of small volumes of cyanobacterial culture supernatant.

3.3.2 Quantitative measurement of extracellular amino acids, peptides, proteins

Table 3.2 describes the quantitative analysis of extracellular nitrogenous compounds of selected cyanobacterial strains harvested at the late exponential/stationary growth phase using the ninhydrin assay system.

Wide variations of extracellular nitrogenous compound release were noted between strains, i.e. in terms of μg leucine equivalents/ml. We have assigned this as extracellular 'protein' - although amino acids and peptides are common in cyanobacterial filtrates, a large proportion of the extracellular nitrogenous materials is in the form of polypeptides (Fogg, 1952; Whitton, 1965; Walsby, 1974). This variation was not attributable to the nitrogen-fixation process since all strains were

grown under conditions of combined nitrogen. Stewart (1964) has shown that compounds apparently similar to those of nitrogen-fixers are released by non-nitrogen fixing forms, and by nitrogen-fixers growing in the presence of assimilable combined nitrogen. The release of extracellular nitrogenous compounds into the medium, irrespective of whether the organisms were growing on elemental or combined N_2, has been found to be substantial, ranging from 5 to 40% of the nitrogen assimilated (Subramanian and Shanmugasundaram, 1986). Our data would confirm this.

When one is interpreting μg 'protein' exported as the percentage dry weight of the cell it is important to consider the growth characteristics of the strain, i.e. it is noteworthy that *Anabaena* CA produces a mucilagenous sheath and large amounts of polysaccharide which increase substantially the cellular dry weight and likewise decrease this percentage value (see Table 3.2).

Variation in extracellular 'protein' release within a species could however, be attributable to the growth phase of the cells (see Table 3.3) with greatest levels of release for *Synechococcus* R2 PCC7942 during the late exponential and stationary phase of growth. This is consistent with the kinetics of the release of extracellular nitrogenous compounds which have been studied in *Anabaena cylindrica*, *Nostoc entophytum*, *Calothrix scopulorum* and *Westiellopsis prolifica* in which the extracellular nitrogen release was reported to be highest during the lag and stationary phases of growth and lowest during the exponential phase (Fogg *et al.*, 1973).

Table 3.2 Estimation of Extracellular Nitrogenous Compounds in cyanobacterial supernatants using a ninhydrin protein assay system

Strain	µg leucine equivalents/ml in culture supernatant	µg 'protein' exported as % dry weight of the cell
Anabaena variabilis	13.1 ± 1.33	12
Anabaena CA	48.3 ± 9.89	1.8
Synechococcus R2 PCC7942	15.89 ± 3.28	5.7
Nostoc PCC6720	27.83 ± 16.24	3.1

Values are the mean of three replications

Table 3.3 Kinetics of extracellular 'protein' release using a fluorescamine assay system in *Synechococcus* R2 PCC7942 under normal and iron-limiting growth conditions

Time (hours)	µg/ml leucine equivalents in culture supernatant		µg/ml leucine equivalents as % dry weight of the cell	
	+Fe	-Fe	+Fe	-Fe
0	0.46	ND	3.06	ND
24	1.38	1.03	4.25	3.97
50	3.72	ND	2.75	ND
96	5.51	ND	1.22	ND
124	5.05	ND	0.78	ND
160	6.89	6.60	0.89	9.42
180	9.18	6.90	1.02	6.22
205	12.40	6.90	1.27	5.43
230	16.80	ND	1.56	ND
275	25.20	8.26	2.40	6.25
300	26.70	8.95	2.60	6.68
350	38.60	9.90	3.60	7.20

ND - not determined

Quantitative data on the release of extracellular protein in other bacteria is limited, but it has been shown in *Myxococcus xanthus* that the quantity of protein secreted did not exceed 4% of the cell protein with the wild-type strain (Nicaud *et al.*, 1984). This organism is unusual in that proteins secreted by *M. xanthus* are not accumulated in the periplasm, as happens in other Gram-negative bacteria, but are secreted into the growth medium. Gram-positive bacteria such as *Bacillus* species are renowned for their extracellular enzyme production and may export grams of protein. This is of a different order of magnitude than cyanobacterial extracellular 'protein' release.

Fogg (1952) showed that at equivalent stages of growth the production of extracellular nitrogenous substances by *Anabaena cylindrica* was considerably increased by deficiency of nutrient elements such as iron. We have shown that growth of *Synechococcus* R2 and *Agmenellum quadruplicatum* PR-6 was severely reduced under iron limitation/ starvation, whereas *Nostoc* sp. MAC grew only slightly slower under iron-limited conditions (see Figs. 3.1, 3.2, 3.3). Addition of the artificial chelator EDDA (0.004% w/v) did not appear to affect the growth rates of the organisms significantly. This is discussed more fully in the next chapter. Analysis of extracellular nitrogenous compounds under such conditions, showed their release to be linked directly to the growth rate and quantitatively much reduced under iron-limiting conditions in actual terms. As a percentage of the cell dry weight however, this figure was increased 2 to 5-fold under iron-limitation for the unicellular strains *Synechococcus* R2 (Table 3.3) and *Agmenellum quadruplicatum* PR-6, though there was no increase observed for *Nostoc* sp. MAC (data not shown).

Figures 3.1-3.3 Graphs of extracellular 'protein', and growth against time, for Synechococcus B2, Anatoc sp. MAC and Synechococcus sp. PCC7002

Figure 3.1 Graphs of a) extracellular 'protein' in terms of μg leucine equivalents ml^{-1} against time (hours), and b) growth (OD_{750}) against time (hours) for Synechococcus B2 grown in i) normal medium (o-----o), ii) normal medium + EDDA (o-----o), iii) chelator-deficient medium (▼---▼)

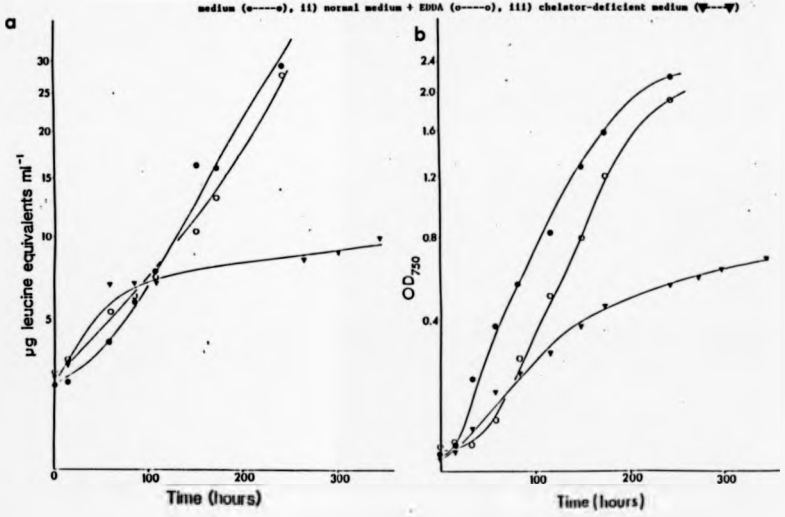


Figure 3.2 Graphs of a) extracellular 'protein' in terms of μg leucine equivalents ml^{-1} against time (hours) and b) growth (OD_{750}) against time (hours) for Hostor sp. MAC grown in i) normal medium (▽—▽), ii) normal medium + EDDA (□—□), iii) chelator-deficient medium (◇—◇)

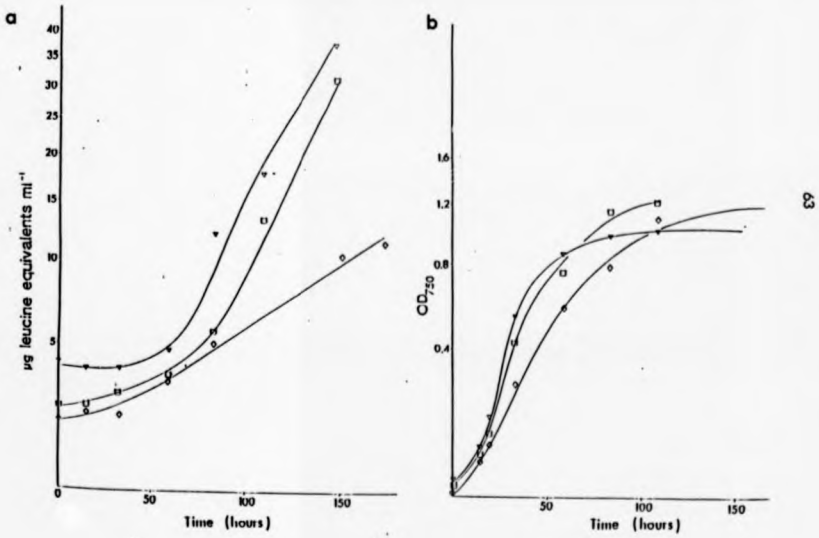
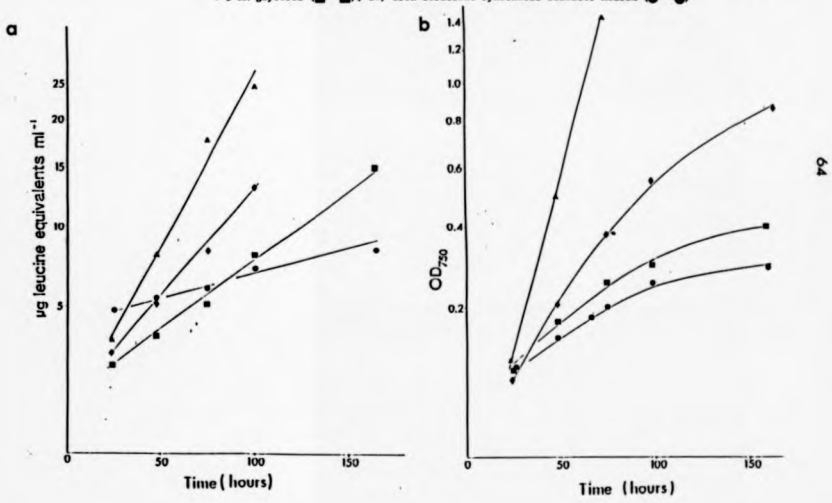


Figure 3.3 Graphs of a) extracellular 'protein' in terms of μg leucine equivalents ml^{-1} against time (hours) and b) growth (OD_{750}) against time (hours) for synechococcus sp. PCC7002 (Agmenellum quadruplicatum PR-6) grown in i) synethetic seawater medium Rippka et al. (1979) + 5 mM glycerol (▲---▲), ii) synethetic seawater medium (◆---◆), iii) iron-deficient synethetic seawater medium + 5 mM glycerol (■---■), iv) iron-deficient synethetic seawater medium (●---●)



3.3.3 Qualitative analysis of cyanobacterial extracellular proteins

The overall size and number of extracellular proteins in concentrated supernatants was characterized electrophoretically using SDS-PAGE. Using this electrophoretic separation technique we have defined a number of criteria for determining truely extracellular proteins: i) that the culture supernatant protein profile is different to the whole cell lysate, and this uses the fact that, ii) electrophoretic separation of cytoplasmic proteins of cyanobacteria are characterised by the presence of the light-harvesting biliproteins, and since these proteins often account for around 30% of the soluble cell protein, and are stable they are a readily detectable feature of a cell lysate. iii) Using a time course of extracellular protein production one can assess if and when cell lysis has occurred.

Analysis of concentrated supernatants of *Synechococcus* R2 and *Nostoc* sp. MAC by SDS-PAGE has shown that *Synechococcus* R2 secretes two major polypeptides of molecular weights 14,500 and 14,200, whilst *Nostoc* sp. MAC secretes a single major polypeptide of 43,000 molecular weight (see figs. 3.4, 3.5). Molecular weights were calculated from a calibration curve, as described in section 2.8. Phycocyanin appears as two subunits, specifically for *Synechococcus* R2 of molecular weight 18,000 and 16,300 after boiling in SDS - comparable to published data (Cohen-Bazire and Bryant, 1982), and such a profile is clearly different from the extracellular one suggesting no lysis has occurred.

A time course analysis of extracellular protein production by *Nostoc* sp. MAC (see Fig. 3.5) shows considerable changes in the number and pattern of extracellular polypeptides as the culture reaches and enters

Figure 3.4 Silver-stained 10-30% (w/v) exponential gradient SDS-PAGE of *Synechococcus* B2 extracellular polypeptides

Lane a - purified phycocyanin (marker of cell lysis). Lanes b-d - extracellular polypeptides (b - normal medium; c - iron-deficient medium; d - phosphate-deficient medium). 10ug protein per track.

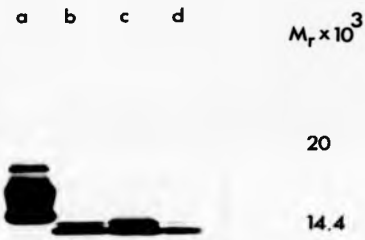
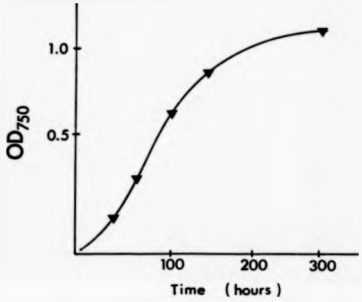
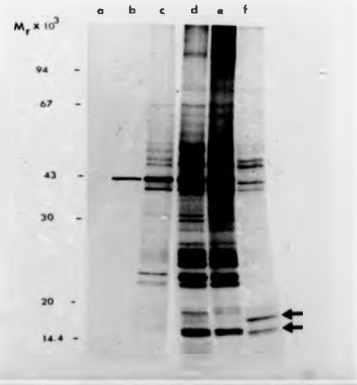


Figure 3.5 Time course of extracellular polypeptide production by *Nostoc* sp. MAC

Silver stained, 10-30% (w/v) exponential gradient SDS-PAGE. Lanes a-e extracellular polypeptides; a - 55 hr; b - 80 hr; c - 105 hr; d - 130 hr; e - 320 hr; f - *Nostoc* sp. MAC cell lysate diluted 1:500. Arrows indicate phycocyanin subunits.





stationary phase, though we are reminded again of the difficulty in establishing the degree of cell lysis. The absence of phycocyanin subunits would suggest that such proteins can be considered secreted.

3.3.4 Analysis of possible functions of extracellular proteins

a) DNAase and RNAase activity

Supernatant from *Nostoc* sp. MAC or *Synechococcus* R2 removed at mid-late exponential phase of growth caused no observable degradation of DNA or RNA, as assessed by agarose gel electrophoresis.

This might be considered surprising in that Wolk (1982) showed only 6 out of 158 strains of heterocyst forming cyanobacteria consistently failed to produce any detectable extracellular deoxyribonuclease activity (though this capacity was shown not to be associated with heterocysts, *per se*). Indeed, Stevens and Porter (1980) indicate that the transformable unicellular cyanobacterium *Agmenellum quadruplicatum* also has the capacity to degrade added DNA even in the presence of 0.1 x SSC (a chelate-containing salt mixture often used to protect DNA from degradation by nucleases), although it was not shown that nucleases were secreted. A potentially transformable strain may have to have some capacity to degrade added DNA, if transformation with covalently closed circular DNA (ccc DNA) is attempted since endonuclease activity could be essential if one or both strands of a linear end must penetrate the cell (see Porter, 1986).

However, *Synechococcus* R2 is naturally competent for transformation, so that rapid degradation of added DNA would be expected to lower observed frequencies of transformation rapidly. It has been shown for

Synechococcus R2 that transformation is totally sensitive to externally addad DNAase (Porter, 1986). Hence there is good reason for the absence of an extracellular DNAase in *Synechococcus* R2. As regards the filamentous strain in question, *Nostoc* sp. MAC, it could be that the active nucleases are cell bound and only released by cell lysis or that the cells secrete nucleases which are rapidly inactivated - especially since Wolk (1982) showed a greater degradation of added DNA by cells, than by supernatant fluids. Truly extracellular DNAases have been reported in only a few bacterial species such as *Serratia marcescens*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

b) Protease activity

Protease activity in supernatants of *Nostoc* sp. MAC and *Synechococcus* R2 was assayed using the methods described in section 3.3.5. It is appreciated that these are only two of the many assays for protease activity. Ammonium sulphate caused interference of the 1% agar gel containing a bovine casein preparation in Tris buffered saline at pH 7.2, and hence the use of protease substrate gel tablets was limited to unconcentrated supernatants. Using both assay techniques, however, no detectable protease activity was observed.

Wolk (1980) showed that *Anabaena variabilis* released an extracellular protease capable of hydrolysing gelatin - though this was demonstrated by streaking cyanobacteria onto substrate-containing agarized growth medium and incubating in the light, and not by incubating solutions of substrate with culture filtrate.

c) Phosphate limitation

Synechococcus R2 and *Nostoc* sp. MAC were grown in 100 ml media containing zero, 2 μM, 4 μM and 20 μM K_2HPO_4 (200 μM in normal media), and supernatants removed and concentrated using ammonium sulphate precipitation before analysis by SDS-PAGE. Since the growth rate is altered by phosphate deprivation (Healey, 1973) the extracellular protein loaded per track was that from an equal cell number, so that any quantitative difference between tracks might be observable. However, no difference, either quantitative or qualitative, of total extracellular polypeptide profile or of the 14,500 or 14,200 polypeptides for *Synechococcus* R2 (Fig. 3.4) or the 43,000 polypeptide for *Nostoc* sp. MAC was observed.

Doonan and Jensen (1980) showed that *Coccochloris peniocytis* has a strong extracellular alkaline phosphatase (PhoA) activity whilst *Anabaena cylindrica* showed little activity associated with the culture medium. A small amount of PhoA enzyme release was also observed by Healey (1973) in *Anabaena variabilis*. None of these workers however, demonstrate unequivocally that such a release was not due in part to cell lysis, and since the active enzyme has been determined for a number of Gram-negative bacteria to be located in some part of the periplasmic space this is an important consideration.

d) Iron-limitation

Synechococcus R2 and *Nostoc* sp. MAC were subject to iron limitation, by growing cells in media containing zero, 0.2 μM, 0.4 μM and 2 μM $FeSo_4.7H_2O$ (20 μM in normal medium), and the extracellular protein profiles examined by SDS-PAGE (concentration of supernatants and sample

loading was as described above). There was no observed change in extracellular protein profile under conditions of iron limitation in *Synechococcus* R2, but *Nostoc* sp. MAC showed a significant increase in production of the 43,000 molecular weight polypeptide with decreasing iron concentration in the medium (see fig. 3.6). The apparent increase in production of this polypeptide under low iron conditions is considerable especially since there is no concomitant increase in total extracellular nitrogenous compound production under iron limitation in *Nostoc* sp. MAC. However, since iron-limitation quantitatively increases extracellular production in *Synechococcus* R2 it is perhaps surprising that we did not observe any qualitative or quantitative difference in extracellular polypeptide as analysed by SDS-PAGE. Perhaps there is a specific increase in production of peptides and amino acids, or other nitrogenous compounds not detectable by SDS-PAGE. The possible iron-binding function of the 43,000 molecular weight polypeptide was investigated using $^{55}FeCl_3$, and Ferene S, a specific stain for iron-binding proteins in polyacrylamide gels (see section 3.2.3).

Nostoc sp. MAC (100 ml culture) was grown in iron deplete media for 7-8 days prior to the addition of $^{55}FeCl_3$ to a final concentration of 2.5 μCi/ml. After a further 5-6 days cells were harvested and periplasmic and cytoplasmic fractions isolated. The culture supernatant was concentrated by ammonium sulphate precipitation. Non-denaturing gel electrophoresis (Hames and Rickwood, 1983) of the isolated fractions followed by fluorography and autoradiography showed a high molecular weight band in the periplasmic fractions of *Nostoc* sp. MAC isolated by osmotic shock or using chloroform (see fig. 3.7). We cannot deduce whether this iron-binding protein corresponds to the 43,000 molecular

Figure 3.6 Effect of iron-limitation on extracellular polypeptide production by *Nostoc* sp. MAC

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE. Tracks f-i extracellular polypeptides; f - zero Fe; g - 0.2 μM Fe; h - 0.4 μM Fe; i - 2 μM Fe. Protein loaded was per 10^8 cells (see text).

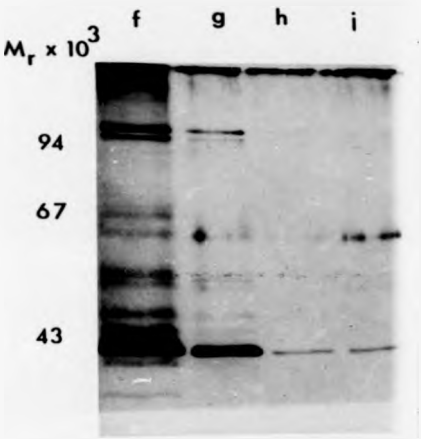
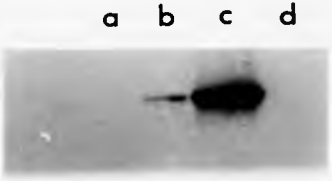


Figure 3.7 Autoradiograph showing periplasmic iron-binding activity by *Nostoc* sp. MAC

4-30% (w/v) non-denaturing PAGE. Lane a - supernatant; lane b - periplasmic fraction (osmotic shock); lane c - periplasmic fraction (chloroform); lane d - cytoplasmic fraction.



weight polypeptide, since SDS-PAGE analysis of the isolated cellular fractions and autoradiography of the fluorographed gel showed no ^{55}Fe polypeptide binding. This would be consistent with the treatment disrupting any iron-protein interaction. It is noteworthy that *Nostoc* sp. MAC possesses two ferradoxins (non-haem iron-containing proteins) in denaturing gel systems approximately 10,500-12,00 molecular weight (Huther, 1977). The absence of ^{55}Fe binding to these cytoplasmic proteins, following ^{55}Fe labelling of cells and SDS-PAGE analysis, agrees with the idea that the SDS treatment has removed any iron-protein association.

Use of Ferene-S failed to produce any complex formation after electrophoresis of *Nostoc* sp. MAC supernatant, periplasmic or cytoplasmic fractions. Whether the strain was specific for the non-haem type iron-binding glycoproteins, e.g. lactoferrin and transferrin described by Chung (1985) is not known.

In a parallel series of experiments *Synechococcus* R2 showed no ^{55}Fe binding after non-denaturing electrophoresis, or complex formation with Ferene-S, for any of the isolated cellular fractions.

It was observed, however, that *Synechococcus* R2 and *Nostoc* sp. MAC produced an extracellular pigment, under both normal and iron-limiting growth conditions during the stationary phase of growth. Aged culture filtrates of *Synechococcus* R2 were lime yellow, and *Nostoc* sp. MAC pale brown. It has already been mentioned that *Anabaena cylindrica* secretes a complex molecule mainly comprising a large pigmented residue, but also containing a substantial peptidic part, which forms a stable complex

with much of the iron supplied in the culture medium (Walsby, 1974). The possibility that similar complexes are formed with the extracellular pigments just described was not investigated . Whether the coloration of the compounds, which complex iron, is incidental to their biological role is not known.

3.3.5 Raising of antibodies to cyanobacterial extracellular proteins

Since we could find no easily assayable function for the extracellular polypeptides under investigation - which may have helped in gene cloning, enzyme localisation studies, etc. - we attempted to raise antibodies to the 14,400 *Synechococcus* R2 polypeptides and the 43,000 molecular weight *Nostoc* sp. MAC polypeptide in order to assist in their recognition. The antigen, the separated polypeptide bands from SDS-PAGE gels, was injected subcutaneously at the back of the neck of a New Zealand white rabbit following the protocol described in Section 4.2.2. Using this scheme no detectable antibody was observed, as measured by Western blotting for either of the polypeptides. It is possible that these polypeptides are not antigenic, but perhaps more plausible is the altered antigenicity associated with using a protein antigen contained within acrylamide - although this method has been successful. Amino acid sequencing, and construction of a synthetic oligonucleotide would be an alternative way of gene cloning, and would give information on the presence of leader sequences and perhaps identify regions of functional homology. This would provide a useful way forward in the area of cyanobacterial protein export.

3.4 Conclusions

Although there is still much discussion on the nature of cyanobacterial extracellular nitrogenous substances, we have shown that high molecular weight polypeptides (>10,000 molecular weight) can be secreted by cyanobacteria, which is in addition to the well documented release of small molecular weight peptides and amino acids.

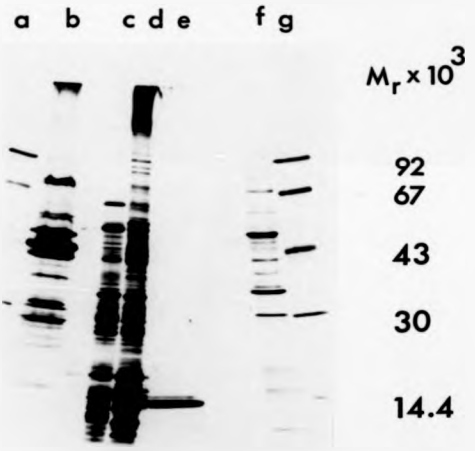
Total extracellular nitrogenous material production is considerable and a maximum level is reached at the late-exponential phase of growth. The time scale of this release may be explained by an initial period of synthesis of extracellular material which is first exported to the periplasm, before its release to the extracellular environment. The synthesis and release of the extracellular enzyme pectate lyase from *Erwinia carotovora* also shows similar kinetics (Hinton, 1986).

Although comparisons of periplasmic and supernatant fractions show markedly different profiles (Fig. 3.8), enzyme localisation data of gene products known to be cytoplasmically located, e.g. β-galactosidase, and periplsmically located, e.g. alkaline phosphatase, was thought necessary to clarify the secretion process. For this reason the expression of foreign proteins in *Synechococcus* R2 and *Nostoc* sp. MAC using plasmids encoding exported gene products was attempted, with the idea this might also tell us whether known secretory sequences, i.e. leader sequences, were functional in cyanobacteria (see Chapter 5).

The molecular biological approach to studying protein secretion in *Nostoc* sp. MAC and *Synechococcus* R2, also attempted the cloning of the genes encoding the observed extracellular polypeptides. The

Figure 1.8 Cellular fractionation of *Nostoc* sp. MAC and *Synechococcus* R2.

Silver stained, 10-30% (w/v) exponential gradient SDS-PAGE. Lanes a and g - molecular weight markers. Lane b - *Nostoc* sp. MAC supernatant; lane c - *Nostoc* sp. MAC periplasmic fraction; lane d - *Nostoc* sp. MAC cytoplasmic fraction; lane e - *Synechococcus* R2 supernatant; lane f - *Synechococcus* R2 cytoplasmic fraction.



unsuccessful attempt at raising antibodies to these proteins which would have made possible the screening of a recombinant λgt11 library, prevented such an approach, however, and hence could give us no information on whether such proteins contained classical signal sequences for their secretion. Transposon mutagenesis and promoter probe utilization have since taken precedence in this line of thought (see Chapters 6 and 7).

Chapter 4

Cyanobacterial Outer Membrane Proteins

4.1 Introduction

So far, we have considered only truly extracellular protein production, i.e. proteins released into the culture supernatant. However, it can be thought that the transport through the cytoplasmic membrane is the primary secretion event. The differences in exoprotein secretion observed between Gram-positive and Gram-negative bacteria appear, therefore, to reside in the physicochemical nature of the cell wall, and not in any fundamental ability to transport proteins across the cytoplasmic membrane. In this sense, the periplasmic and outer membrane proteins of the Gram-negative bacteria have been secreted but because of the cell wall restraint, remain associated with the cell (see also Section 1.3).

Investigation of cyanobacterial cell envelopes (see Section 4.1.1) provide information as to whether the extracellular polypeptides previously described were outer membrane proteins sheared away from the cell wall, whilst their characterization still allowed examination of the secretion process. The known involvement of outer membrane proteins as receptors for specific molecules might shed light on the iron uptake systems of these organisms, and provide a more functional evaluation of cyanobacterial exported proteins.

4.1.2 Cyanobacterial cell walls

The cell wall is involved in determination and maintenance of cell shape, and its synthesis is correlated with the process of growth, division and differentiation of cells. The export and import of substances can be controlled by the cell wall. Receptors for cyanophage have been localised in the outer membrane.

The cell envelope of cyanobacteria comprises three distinct layers: cytoplasmic/membrane, the cell wall (pepitdoglycan layer plus outer membrane), and an external layer which may be a sheath, or slime (for review see Jurgens and Weckesser, 1985).

Whilst an outer membrane is found in all cyanobacteria, the possibility that cyanobacteria possess a cell wall structure comprising features of both a Gram-negative and a Gram-positive organism has been put forward (Jurgens and Weckesser, 1985). Structural analysis of the peptidoglycan from the unicellular cyanobacterium *Synechocystis* PCC6714 has shown the presence of a polysaccharide covalently bound via phosphodiester bridges to the peptidoglycan. The degree of cross-linkage of the peptidoglycan was about 56%, within the range found with peptidoglycan from Gram-positive bacteria. These two features, together with the presence of a relatively thick peptidoglycan layer (approx. 10 nm on average), suggests that peptidoglycan of *Synechocystis* PCC6714 is comparable more with the Gram-positive bacteria.

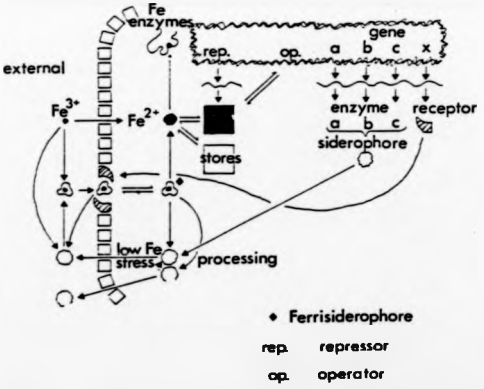
In the few strains that have been studied, lipopolysaccharide, lipids and proteins have all been shown to be constituents of the cyanobacterial outer membrane. Only few proteins dominate polypeptide patterns of cell wall fractions and their molecular weight lie in a range higher than that of porins of Gram-negative bacteria (Resch and Gibson, 1983; this study). A more unusual feature of cyanobacterial cell walls is the presence of carotenoids in the outer membrane (Omata and Murata, 1984; Jurgens and Weckesser, 1985). Their function in the outer membrane is unknown, but protection against high light intensities or a structural function may be suggested (see Siefermann-Harms, 1987).

4.1.3 Bacterial iron uptake systems

Iron is required as an essential trace element for bacterial growth and its role in such processes as electron transport and nitrogen fixation is well established. In an aerobic external environment, however, iron exists in an insoluble form as a ferric hydroxide polymer. To assimilate iron, bacteria have developed high and low affinity iron-uptake systems. The high affinity pathway is comprised of two parts - relatively low molecular weight (500-1000 daltons) iron-chelating compounds called siderophores, and a membrane-bound system for transport and utilization of the chelated iron (see Neilands and Nakamura, 1985). All components of the high-affinity pathway are induced by growth of the organism at low levels of iron (ca. <1 μM). Siderophores form complexes of high affinity and specificity with Fe^{3+}. A number have been characterised (reviewed by Neilands, 1984). The low affinity process is non-specific, does not require specific carriers and appears to be constitutive. A schematic model of low and high affinity iron assimilation pathways is depicted in Figure 4.1. In *Escherichia coli*, 4 different iron uptake systems have been characterized, i) the iron (III) enterobactin receptor, ii) the ferrichrome receptor, iii) the iron (III)-citrate and iv) the iron (III)-aerobactin receptor. Plasmids as well as chromosomal DNA, are involved in specifying elements of the ferric aerobacterin transport system in enteric bacteria. Detailed molecular studies have now been reported for the aerobactin (Lorenzo *et al.*, 1987) and enterobactin (Nahlik *et al.*, 1987) iron-uptake gene clusters.

Figure 4.1 Schematic representation of low and high affinity iron assimilation

In the former, environmental Fe(III) crosses the cell envelope not coordinated to specific ligands and without mediation by membrane-associated receptors. The high affinity pathway is comprised of Fe(III)-specific carriers (circles), termed siderophores, and receptors. In *E. coli* 4 mechanisms of siderophore-mediated iron uptake are possible. Only the iron of the ferrisiderophore may be deposited in the envelope, or conversely, the intact complex may be incorporated. Of the latter, the iron may be removed without processing of the ligand; conversely, the ligand may be either reversibly or irreversibly (broken circles) processed. (From Neilands, 1984).



4.1.4 Effects of iron starvation and iron restoration on the ultrastructure and physiology of cyanobacteria

Effects of iron deficiency have been reported for two unicellular cyanobacteria *Synechococcus* R2 and *Agmenellum quadruplicatum* PR-6 (Hardie *et al.*, 1983; Pakrasi *et al.*, 1985). Iron starved cells have decreased amounts of chlorophyll and phycobiliproteins, and show alterations in the composition of their photosynthetic membranes. The chlorophyll absorption peak of iron-starved cells, at 672 nm, is 7 nm blue-shifted from its normal position at 679 nm, and LDS-PAGE analysis has shown iron-deficient membranes substantially lack the high molecular weight chlorophyll-protein complexes. There is also a large increase in glycogen storage granules. Under iron stress cells adjust their ferredoxin levels. At levels of iron below 2 μM, there is a rapid loss of ferredoxin, but a similar sharp increase in the amount of flavodoxin occurs, a flavoprotein that contains no iron group. The replacement of ferredoxins with flavodoxin is important in that it allows physiological processes to proceed even under periods of iron stress.

Iron restoration causes an immediate utilization of the glycogen granules - presumably a carbon and energy source, and the resumption of pigment synthesis, with phycocyanin preferentially synthesized as compared to chlorophyll over the first 6 hr. By 24 hr, the ratio of phycocyanin and chlorophyll is similar to that found in normal cells and the chlorophyll maxima at 679 nm has been restored. This perhaps correlates with the sequential changes in the organization of the thylakoid membrane of cells recovering from iron starvation. There are two distinct stages in the iron-induced restoration of the photosynthetic membranes: different components of the thylakoid

membranes are synthesised and then inserted into membranes during the first 12 hr and new membrane synthesis and cell division begin between 12 and 24 hr.

4.1.5 Iron chelation and uptake by cyanobacteria

Cyanobacteria make use of a wide variety of chelators to solubilise the iron in their environment, not all of them synthesized by the cyanobacteria themselves. These range from the relatively non-specific (but widespread) humic substances to the highly specific siderophores. The content of iron in cyanobacteria is some 3-20 times higher than in other bacteria whereas levels of their trace metals are similar to those found in bacterial cells (Jones *et al.*, 1978).

Several different techniques have been utilized to investigate the occurrence of siderophores in cyanobacteria. No single method is a universal indicator for the presence or absence of these compounds, therefore their occurrence has been investigated using a combination of approaches. Biological tests include, i) the ability to grow on iron - and/or chelator-deficient media, ii) the ability to promote growth in siderophore auxotrophs and iii) the ability to moderate copper toxicity.

Lange (1974) found six cyanobacteria able to grow in defined media in the absence of external chelator (EDTA). The chelators produced by these cyanobacteria need not necessarily be siderophores, since at least three species of cyanobacteria produce only low affinity chelators in response to growth on chelator deficient media (McKnight and Morel, 1979).

Arthrobacter flavescens JG-9, an obligate auxotroph for hydroxamate siderophores (Neilands, 1984) is the organism commonly employed in the second bioassay. Estep *et al.* (1975) showed cyanobacterial mats from various marine ecosystems contained JG-9 growth promoting activity.

Several species of cyanobacteria produce strong copper complexing agents that reduce the toxicity of this free metal ion (McKnight and Morel, 1979). The production of these compounds was greatly stimulated by iron limitation, and the iron-chelator complex was significantly more stable than the copper-chelator complex. There is now strong evidance to suggest that these strong copper-complexing agents are hydroxamate siderophores (Clarke *et al.*, 1987).

Two chemical tests have been utilized for detection of siderophore production - the Arnow test for catechols and the Czaky method for bound hydroxylamines. The Arnow test has consistently proved negative when applied to various cyanobacteria, whilst Czaky positive material has been reported for *Agmenellum quadruplicatum* PR-6, *Anabaena flos-aquae* and in two other isolates of *Anabaena*. Boyer *et al.* (1987) has summarized the data concerning siderophore activity in cyanobacteria.

The only siderophore which has been structurally characterized is schizokinen, a dihydroxamate produced by *Anabaena* sp. strain ATCC 27898 (Simpson and Neilands, 1976). This is a member of the citrate-hydroxamate family of siderophores including aerobactin and arthrobactin. Lammers and Sanders-Loehr (1982) showed that ATP served as an energy source for the cellular uptake of ferric schizokinen - light-driven transport was inhibited by uncouplers of the transmembrane

proton gradient, and by ATPase inhibitors, whilst transport in dark adapted cells was additionally blocked by inhibitors of respiration.

4.2 Materials and Methods

4.2.1 Isolation of cell walls and outer membranes from cyanobacteria

A carotenoid-containing cell wall fraction was isolated using a method based on that described by Resch and Gibson (1983). Harvested cells (100 ml) were resuspended in 4 ml 10 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid) pH 7.2 and passed twice through a French Pressure cell at 24,000 psi. (Alternative methods of cell breakage, such as freeze-thawing or sonication, significantly affected the integrity of the membrane preparation). Whole cells were removed by spinning at 3000 rpm in an MSE Chillspin for 5 min. The supernatant was layered onto a discontinuous 50-85% (w/v) sucrose gradient in 10 mM HEPES pH 7.2 in 17 ml or 38 ml polyallomer centrifuge tubes and centrifuged at 4°C for 18 hr at 25,000 rpm in a Beckman SW27 rotor using a Beckman L8 ultracentrifuge. The gradients were fractionated after puncturing the bottom of the tubes.

Triton X-100 insoluble cell wall fractions were prepared as follows. The cell wall fraction (1 ml) obtained after sucrose density gradient centrifugation, was diluted with 7 ml 10 mM HEPES, 10 mM $MgCl_2$ containing Trion X-100 at a final concentration of 2%. After vortexing, samples were loaded into 10 ml polycarbonate tubes and centrifuged at 45,000 rpm at 4°C for 90 min using a 10 x 10 ml rotor and MSE Prepsin 65. After centrifugation, the supernatant was removed, and the Triton X-100 insoluble pellet resuspended in 500 µl 10 mM HEPES pH 7.2.

Triton X-100 insoluble cell wall fractions were also prepared from whole cell lysates, after breakage of cells in a French pressure cell at 24,000 psi, using the method described above, except that the Triton extraction procedure was repeated twice.

Cytoplasmic membranes, thylakoid membranes, cell walls and soluble fractions were also isolated using a method described by Omata and Ogawa (1986).

4.2.2 Antibody production

Purified cell walls from *Synechococcus* R2 (isolated as described above) were boiled in 1% SDS and the preparation used as antigen to inject New Zealand white rabbits. The immunization schedule was similar to that described by Hames and Rickwood (1983). Prior to the first antigen injection a pre-immune serum sample was removed from the rabbit.

Day 0: 150 μg antigen in 1 ml 0.05 M Tris pH 6.8 was emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories Ltd.) and injected subcutaneously at the back of the neck.

Day 15: A second injection of antigen (150 μg) was emulsified with an equal volume of incomplete Freund's adjuvant and injected subcutaneously as above. This injection was repeated 5 days later.

Day 30: 25 ml of blood was removed, and the antisera stored at -20°C in 1 ml portions prior to use in Western blotting.

4.2.3 Western blotting

Proteins separated by SDS-PAGE were transferred to nitrocellulose using a Biorad transblot system at 300 mA for 3 hr in buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol. Ponceau S (0.5% w/v in 5% TCA) was used to visualize protein on the nitrocellulose filter. The filter was incubated in dye solution for 10 min with shaking, and unbound dye removed by washing with water. Filters were transferred to PBS solution to remove all remaining dye.

The Amersham biotin-streptavidin detection system was then used for immunoscreening and optimized as follows. After blotting, filter(s) were blocked in 50 ml 0.14 M NaCl, 0.02 M sodium phosphate pH 7.3 (PBS) containing 3% BSA (Pentax fraction V) with shaking for 15 min at room temperature. Primary antibody was added to the appropriate dilution (1:100 - 1:250) and filters incubated for 1 hr. After five 5 min washes in PBS, the filter(s) were transferred to 50 ml 1% BSA in PBS, 25 µl biotinylated protein A was added, and the filter(s) incubated for a further 60 min. This was followed by five 5 min stringent washes in PBS containing 1% BSA in PBS before adding 125 µl streptavidin-peroxidase complex and incubating at room temperature for 30 min with shaking. Before colour development, the filters were subject to three 5 min washes in PBS + 1% Triton X-100 and then transferred to 50 ml of a solution containing 0.5 mg ml^{-1} diaminobenzidine (Sigma), 0.03% (w/v) $NiCl_2$ in PBS. 33 µl of 30% hydrogen peroxide was added to initiate the staining reaction and the filters shaken until the desired intensity of band was obtained.

An alternative screening procedure was also used which was found to be

more sensitive and reliable. Peroxidase conjugated goat anti-rabbit IgG was used as secondary antibody, and O-dianisidine (Sigma) as the colour reaction reagent. After transfer, filters were blocked in 50 ml of TBS solution (150 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing 5% BSA for 1 hr. Primary antibody was added to the appropriate dilution (1:1000 - 1:1500) and filters incubated for 1 hr. After six 10 min washes in TBS containing 1% Triton X-100 filters were transferred to a solution of 1% Triton X-100 in TBS containing secondary antibody (peroxidase conjugated goat anti-rabbit IgG) 1:10,000 dilution, and left shaking for 1 hr. After five 10 min washes in TBS + 1% Triton X-100 and one 10 min wash in TBS, filters were transferred to a solution containing 160 ml 10 mM Tris pH 8.0, and 1 ml O-dianisidine solution added (14 mg O-dianisidine in 1 ml methanol). 50 μl hydrogen peroxide was then added to initiate the staining reaction.

4.2.4 Screening of the λgt11 library with antibody probes

Escherichia coli strain Y1090 was grown to stationary phase in 5 ml LB medium. To 0.1 ml of the Y1090 culture, 0.1 ml of SM buffer (5.8 g NaCl, 2 g $MgSO_4.7H_2O$, 50 ml 1 M Tris-HCl pH 7.5, 5 ml 2% gelatin per litre) containing 2 x 10^4 pfu of λgt11 library was added and incubated at 37°C for 10 min for phage adsorption. The infected bacteria were added to 4 ml of molten Luria soft agar and plated on LB plates containing 50 μg ml^{-1} ampicillin. The plates were kept at 42°C for 3-4 hr and dry nitrocellulose filters that had been presoaked with 10 mM isopropylthiogalactoside, were overlayed on the agar. The plates were shifted to 37°C and the incubation continued for a further 3-4 hr until individual plaques were clearly visible. These plates could be stored at 4°C overnight if necessary.

Polyclonal antibodies often contain components which bind to antigens normally produced by *E. coli*. In order to avoid the high background of signals produced by this binding activity, antibody was preabsorbed against a bound bacterial lysate. Six plate lysates were made of Y1090 with non-recombinant λgt11 as described above for the λgt11 library, except that the plates were incubated overnight at 37°C. 5 ml SM buffer was then added to each plate and the plates left at room temperature for 3-4 hr. A nitrocellulose filter was then incubated with this lysate at 37°C for 2 hr, before transferring it to a solution (25 ml) containing PBS, 0.02% azide 3% BSA and incubating for a further 2 hr at 37°C. Appropriately diluted antibody, in PBS + 2% BSA was then incubated with this filter at 37°C overnight. The antibody solution was then ready for use in screening. The nitrocellulose filters were then carefully removed from the plate and incubated for 1 hr in PBS containing 5% BSA. The filters were then incubated for 1 hr in primary antibody (pre-treated as above) and given six 10 min washes with PBS + 1% Triton X-100 before incubating for 2 hr in ^{125}I-labelled protein A (0.1-0.5 μCi/ml). Filters were then subject to three 30 min washes in PBS + 1% Triton X-100 (or until counts in the wash were below 20 cpm) and positive plaques identified by autoradiography.

4.2.5 Detection of siderophore activity

The method of Schwyn and Neilands (1987) was used for the detection of siderophore, this is based on the use of the complex chrome azurol S (CAS)/iron III/hexadecyltrimethyl ammonium bromide (HDTMA). When a strong chelator removes the iron from the dye, its colour turns from blue to orange.

A 6 ml volume of 10 mM HDTMA solution (Sigma) was placed in a 100 ml volumetric flask and diluted with water. A mixture of 1.5 ml 1 mM $FeCl_3.6H_2O$, 10 mM HCl and 7.5 ml 2 mM aqueous CAS solution (Sigma) was slowly added under stirring. A 4.307 g quantity of anhydrous piperazine was dissolved in water and 6.25 ml of 12 M HCl was carefully added. The buffer solution was rinsed into the volumetric flask and the volume made up to 100 ml. 4 mM 5-sulfosalicylic acid was added to this solution to make the CAS shuttle solution.

A 0.5 ml aliquot of supernatant was mixed with CAS assay solution and the absorbance measured at 630 nm after reaching equilibrium. A blank was prepared using normal growth medium. The CAS shuttle solution was used when iron exchange was slow. Here, the absorbance at 630 nm was measured within 6 hr because the blue dye precipitated during longer storage.

4.2.6 Routine spectrophotometry

Absorption spectra were obtained on a Pye Unicam SP1700 double beam spectrophotometer at room tempertaure.

4.3 Results and Discussion

4.3.1 Isolation of a cell wall fraction, and characterization of outer membrane proteins from *Synechococcus* R2

A cell wall fraction containing outer membrane plus peptidoglycan was isolated from *Synechococcus* R2 using a modification of the method described by Rasch and Gibson (1983) - see Section 4.2.1. In this modified isolation procedure, a linear sucrose gradient step was

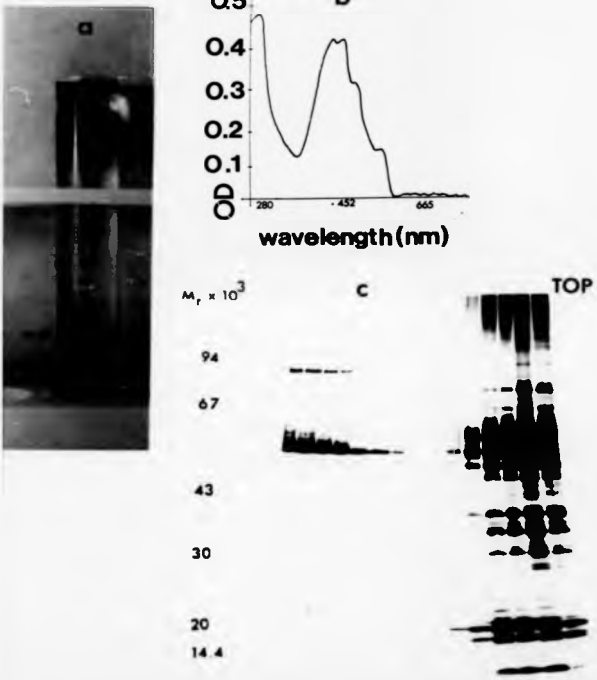
replaced with a discontinuous sucrose gradient system. This produced sharper banding of the cell wall fraction.

Membrane preparations centrifuged on discontinuous 50-85% (w/v) sucrose gradients prepared in 10 mM HEPES (pH 7.2) showed a similar pigment distribution (see Fig. 4.2). A broad blue-green band occurred at sucrose densities of less than 60% (w/v). This contained detached phycobiliproteins and disrupted thylakoid membranes containing chlorophyll, as assessed by spectrophotometry, and SDS-PAGE (Fig. 4.2). At sucrose densities of 75-80% (w/v), equivalent to a buoyant density of 1.28 g cm^{-3}, a yellow band(s) occurred. The presence of more than one band in this region possibly reflected the degree of integrity of the cell wall fraction; a loss of peptidoglycan reduced the buoyant density of a proportion of the cell wall fraction and gave rise to two bands. Both fractions showed similar outer membrane protein profiles. These dense pigmented membrane fractions are characteristic of the cell walls of cyanobacteria (Omata and Murata, 1984; and see Section 4.1.2). The peptidoglycan associated with the outer membrane gave this fraction a high bouyant density, and allowed separation from the inner or cytoplasmic membrane.

The absorption spectrum of the isolated cell wall fraction (see Fig. 4.2) showed absorption maxima at 430 nm, 452 nm and 488 nm - indicative of carotenoids, whereas little, or no contamination of chlorphyll A (665 nm) or phycobiliproteins (565 nm, 620 nm) was detectable in these preparations. The presence of carotenoids in cell wall fractions of cyanobacteria is now generally accepted (see Jurgens and Weckesser, 1985). Treatment of cell wall fractions with 1% Triton X-100, which

Figure 4.2 *Synechococcus* R2 outer membrane proteins

a) Cell wall fraction (arrowed) from *Synechococcus* R2 isolated by sucrose density gradient centrifugation 50-85% (w/v). b) Absorption spectrum of isolated cell wall fraction. c) Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of fractions from the sucrose gradient shown in a).



selectively solubilizes the cytoplasmic membrane of Gram-negative bacteria (Schnaitman, 1981), followed by sucrose density gradient centrifugation yielded material which still contained carotenoids (Rasch and Gibson, 1983; this study). This suggested that carotenoids were a true component of the cell wall and not due to contamination by cytoplasmic membranes.

Under normal growth conditions the major polypeptides of the *Synechococcus* R2 carotenoid-containing outer membrane fraction, after solubilization at 100°C, corresponded to 78,000 molecular weight, together with a large composite band at 52,000 molecular weight (see Fig. 4.2). This broad band was resolved into 2 or 3 components (see Fig. 4.3). The absence of polypeptides of approx. 14,400 molecular weight in the outer membrane, suggested that the extracellular polypeptides described in the previous chapter were not simply proteins sheared away from the cell wall. This is supported by the absence of polypeptides of approx. 52,000 molecular weight in the culture medium. We might infer then, that the cell envelope was intact at the time of isolation of the extracellular protein fraction.

4.3.1.1 Effect of solubilization temperature on the electrophoretic mobility of outer membrane proteins of *Synechococcus* R2

Distinct changes in the SDS-PAGE polypeptide pattern of outer membrane proteins occurred when the temperature conditions used for sample solubilization were altered (see Fig. 4.4). At temperatures below 45°C, major amounts of the total protein applied were retained in the upper part of the gel and the major proteins (approx. 52,000 molecular weight) were completely absent. Two polypeptides of 70,000 and 67,000 molecular

Figure 4.3 Western blot using antibody (1:100 dilution) raised against a cell wall fraction of *Synechococcus* R2, and the Amersham biotin-streptavidin detection system for immunoscreening

Lane a - *Synechocystis* sp. PCC6308 outer membrane proteins (OMP), normal medium; lane b - *Synechococcus* R2 OMP, normal medium + 10 mM sucrose; lane c - *Synechococcus* R2 OMP, normal medium; lane d - *Synechococcus* R2 OMP, normal medium + EDDA 0.004%; lane e - *Synechococcus* R2 OMP, chelator-deficient medium; lane f - *Synechocystis* sp. PCC6803 OMP, normal medium.

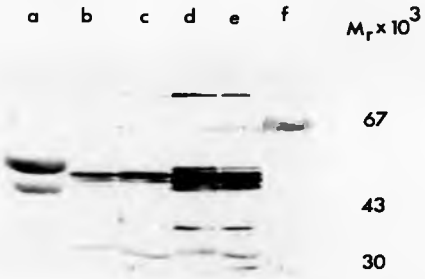
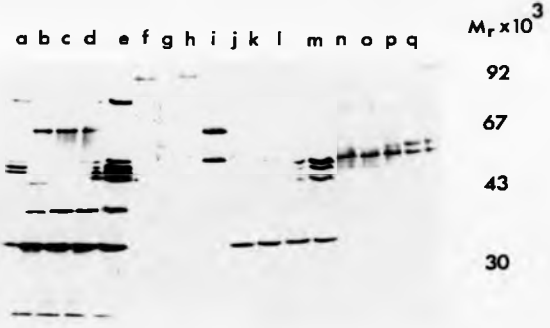


Figure 4.4 Effect of solubilization temperature on the electrophoretic mobility of cyanobacterial outer membrane proteins

Silver-stained 10-30% (w/v) exponential gradient SDS-PAGE. Lane a - *Synechococcus* R2 cell wall fraction (normal medium) solubilized at 100°C; lanes b-e *Synechococcus* R2 cell wall fraction (chelator-deficient medium) solubilized at: lane b - 25°C; lane c - 50°C; lane d - 65°C; lane e - 100°C. Lanes f-i *Synechocystis* sp. PCC6803 cell wall fraction (chelator-deficient medium) solubilised at lane f - 25°C; lane g - 50°C; lane h - 65°C; lane i - 100°C. Lanes j-m *Synechocystis* sp. PCC6308 cell wall fraction (chelator-deficient medium) solubilised at: lane j - 25°C; lane k - 50°C; lane l - 65°C; lane m - 100°C. Lanes n-q *Nostoc* sp. MAC cell wall fraction (chelator-deficient medium) solubilized at: lane n - 25°C; lane o - 50°C; lane p 65°C; lane q - 100°C.



weight were visible at lower temperatures, but as the temperature was raised these became absent, and at 100°C the major proteins were most prominent. Omata and Murata (1984) described major polypeptides of a cell wall fraction of *Synechocystis* sp. PCC6714 that were solubilized with sodium dodecyl sulphate only at high temperature. However, the mobility of several polypeptides from *Synechococcus* R2 including a 35,000 molecular weight polypeptide, induced under conditions of iron limitation (see Section 4.3.1), were not altered with varying solubilization temperatures. This suggests that only particular outer membrane proteins are affected in this way.

Nakamura and Mizushima (1976) showed that the major outer membrane proteins of *E. coli*, which function as porins, are heat-modifiable, i.e. when the membrane preparation was heated in SDS solution, the position and number of protein bands in SDS-PAGE were altered. This was attributable to a change in protein conformation from a β-structure to a α-helix upon heating. It is tempting to correlate the physical property of a resistance to denaturation by SDS at temperatures below 85-100°C, of the proteins described here, with their functioning as porins. Recently, Benz *et al.* (1987) demonstrated pore forming activity of the major outer membrane protein of *Rhodobacter capsulatus* in lipid bilayer membranes. This protein was heat-modifiable - it had an apparent molecular weight of 69,000 when it was solubilized below 60°C, but at temperatures above 70°C the apparent molecular weight decreased to 33,000. Pore formation by an outer membrane protein of the cyanobacterium *Anabaena variabilis* has also been demonstrated (Benz and Bohme, 1985), but they provided no data as to whether the major outer membrane proteins were heat-modifiable. Although we have observed

changes in the electrophoretic mobility of the major outer membrane proteins of *Synechococcus* R2 with varying solubilization temperature, further work, such as permeability measurements in reconstituted phospholipid vesicle systems, is required before we can infer with confidence that these proteins function as porins.

4.3.1.2 Effect of changes in osmolarity on the pattern of outer membrane proteins in *Synechococcus* R2

Synechococcus R2 was grown in Allen's medium containing 10 μM, 100 μM, 1 mM, 10 mM or 100 mM NaCl, and cell wall fractions isolated as described previously. The pattern of outer membrane proteins was investigated under the various salt concentrations by SDS-PAGE. No qualitative change was observed under these conditions (see Fig. 4.5). However, *Synechococcus* R2 grown in Allen's medium containing 1 mM, 5 mM, 10 mM, 50 mM or 100 mM sucrose showed an alteration in the pattern of outer membrane proteins at sucrose concentrations up to 50 mM. At low sucrose concentrations a polypeptide of 50,000 molecular weight was present which was absent in normal medium (see Fig. 4.6). The regulation of outer membrane proteins by osmolarity is well documented in *E. coli* (see Nara *et al.*, 1986). The outer membrane of *E. coli* contains two major porin proteins, OmpF and OmpC. Although these two proteins are similar with respect to functional and structural properties, expression of the genes that code for OmpF and OmpC, are regulated in opposite directions by the osmolarity of the medium. As the osmolarity increases, OmpF synthesis is depressed and OmpC synthesis is enhanced. The differential change in synthesis of these proteins presumably protects the cell against such a high osmolarity. This could be accomplished by either decreasing the influx of harmful components or

Figure 4.5 Effect of salt concentration on the pattern of outer membrane proteins in *Synechococcus* R2

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE. Lanes a, b - 10 μM NaCl; lanes c, d - 100 μM NaCl; lanes e, f - 1 mM NaCl; lanes g, h - 10 mM NaCl; lanes i, j - 100 mM NaCl; lanes k, l - normal medium. Each pair are consecutive sucrose density gradient fractions.

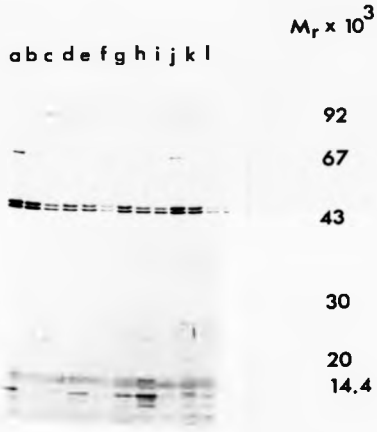
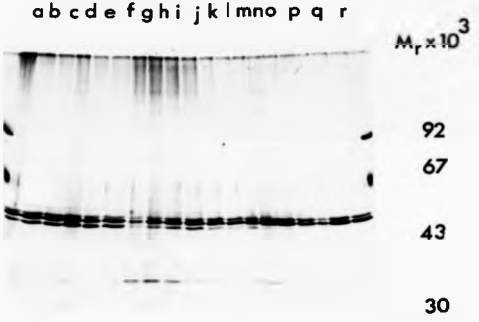


Figure 4.4 Effect of sucrose concentration on the pattern of outer membrane proteins in *Synechococcus* R2

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of three consecutive sucrose density gradient fractions of cell walls isolated from *Synechococcus* R2 grown in Allen's medium + sucrose at the stated concentration. Lanes a-c - 1 mM sucrose; lanes d-f - 5 mM sucrose; lanes g-i - 10 mM sucrose; lanes j-l - 50 mM sucrose; lanes m-o - 100 mM sucrose; lanes p-r - normal medium.



waste products, or to facilitate the influx of certain nutrients.

Our observations are confusing in that changes in salt concentration in the medium had no detectable effect on outer membrane protein pattern. In *E. coli* both NaCl and sucrose affect this pattern, although at higher concentrations (300 mM NaCl or 600 mM sucrose). Higher salt concentrations were not used in our investigation. The change in outer membrane protein pattern after the addition of low concentrations of sucrose, but not at 100 mM sucrose, is difficult to interpret. Variation in growth rate was thought not involved since the growth (OD_{750}) of the cultures under varying sucrose concentrations was not altered. It could be at low sucrose concentrations an uptake mechanism is induced, which is switched off at saturating levels of sucrose. These observations might then, not reflect an 'osmolarity factor' but be involved solely with nutrient uptake. Alternatively, these results may simply reflect a change in the structural integrity of the membrane under the various conditions, or the instability of particular outer membrane proteins in high sugar solutions. Clearly, more work in this area is required - especially to determine whether *Synechococcus* R2 induces a specific transport system to take up this sugar. It is doubtful that *Synechococcus* R2 utilizes sucrose, especially since this organism cannot grow photoheterotrophically.

4.3.1.3 Effect of procaine, on the pattern of outer membrane proteins in *Synechococcus* R2

In *E. coli*, the local anaesthetic procaine, at sublethal concentrations, blocks the expression of a particular set of exported protein genes (*ompF*, *ompC*, *phoA* and *malE*). This control is mediated through the *envZ*

gene product, part of the *OmpB* operon - which is involved in porin regulation (see Villarejo and Cass, 1984). Whether procaine caused a similar change in expression of the major outer membrane proteins of *Synechococcus* R2 was investigated.

Procaine was lethal to *Synechococcus* R2 at concentrations above 100 μM in liquid medium (see Fig. 4.7). This is in contrast to *E. coli*, in which the sublethal concentration used in experiments was 20 mM in nutrient broth and 50 mM in agar. Outer membrane proteins isolated from *Synechococcus* R2 grown in 1 μM, 10 μM and 50 μM procaine showed no difference in their SDS-PAGE profile under these conditions (data not shown). The effect, if any, on the expression of alkaline phosphatase under the same conditions, was not investigated.

4.3.1.4 Effects of nutrient limitation on the pattern of outer membrane proteins in *Synechococcus* R2

a) Phosphate limitation

Growth of *Synechococcus* R2 in phosphate-deficient, or phosphate-limiting medium (2 μM-20 μM phosphate in the medium; 200 μM in normal medium) caused no alteration in the SDS-PAGE pattern of the major outer membrane proteins (approx. 52,000 molecular weight). Minor changes observed, were the induction of polypeptides of 32,000 molecular weight, and one over 100,000 molecular weight (see Fig. 4.8). Phosphate-regulated outer membrane proteins have been identified in other bacteria, including the PhoE proteins of *E. coli*, *Salmonella typhimurium* and *Enterobacter cloacae*, and a 36,000 molecular weight protein of *Klebsiella aerogenes*. All have been shown to form weakly anion-selective channels consistent with their presumed roles in phosphate acquisition. More recently,

Figure 4.7 Effect of procaine concentration on the growth of *Synechococcus* R2.

Graph of growth (OD_{750}) against time (hours) for *Synechococcus* R2 grown in a) normal medium □----□; b) normal medium + 10 μM procaine ■----■; c) normal medium + 30 μM procaine ▼----▼; d) normal medium + 50 μM procaine ◆----◆; e) normal medium + 100 μM procaine ▲----▲; f) normal medium + 500 μM procaine ●----●.

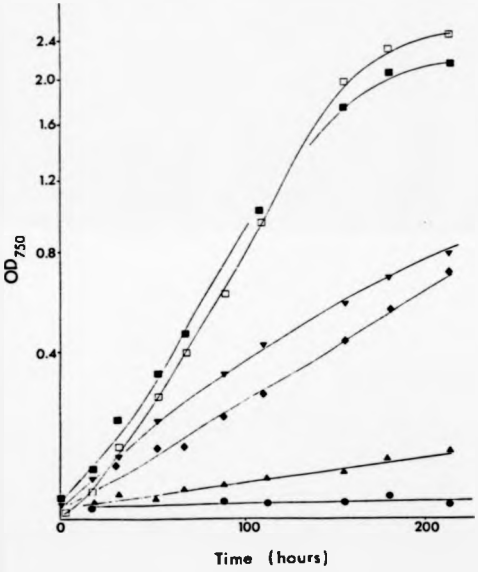
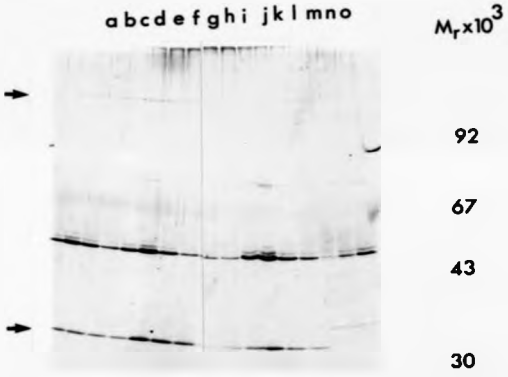


Figure 4.8 Effect of phosphate limitation on the pattern of outer membrane proteins in *Synechococcus* R2

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of three consecutive sucrose density gradient fractions of isolated cell walls from *Synechococcus* R2 grown in medium containing: lanes a-c - zero phosphate; lanes d-f - 2 μM phosphate; lanes g-i - 4 μM phosphate; lanes j-l - 20 μM phosphate; lanes m-o - 200 μM phosphate (normal medium). Arrows indicate induced proteins.



Poole and Hancock (1986) showed that many bacteria from membranes of the families *Enterobacteriaceae* and *Pseudomonaceae* produced phosphate-starvation induced outer membrane proteins which cross-react immunologically. These fell into 2 classes based on the molecular weight of heat-dissociated monomers (45,000 to 50,000 or 36,000 to 39,000 molecular weight), and the detergent conditions.

b) Calcium limitation

Growth of *Synechococcus* R2 in calcium deficient medium caused no observable change in the outer membrane protein profile. Similarly, no change in the absorption spectrum of isolated cell walls was apparent (see Fig. 4.9). Brand and Sheng (1987) showed that at least 3 polypeptides (21,000, 33,000 and 48,000 molecular weight) were diminished in isolated photosynthetic membranes of *Synechococcus* R2, but their investigations were limited to this membrane system.

c) Magnesium limitation

Growth of *Synechococcus* R2 in magnesium deficient growth medium caused various changes in the outer membrane protein profile. Polypeptides of 80,000, 67,000, 62,000, 50,000, 28,000 an 25,000 molecular weight were observed to be induced in cell wall fractions purified from sucrose density gradients (see Fig. 4.10). However, Triton X-100 insolubilized cell wall fractions, from magnesium deficient medium, showed a profile considerably reduced in these apparently outer membrane induced proteins (see Fig. 4.11). The polypeptide profile of fractions from further up the sucrose gradient showed similar induced polypeptides to those in the cell wall fraction, which questions their true location. Even so, these polypeptides were specificially induced under conditions of magnesium

Figure 4.9 Absorption spectra of isolated cell wall fractions from Synechococcus R2

Grown in; i) normal medium ——— ; ii) chelator-deficient medium .....; iii) magnesium-deficient medium - - - - -; and iv) calcium deficient medium — — —.

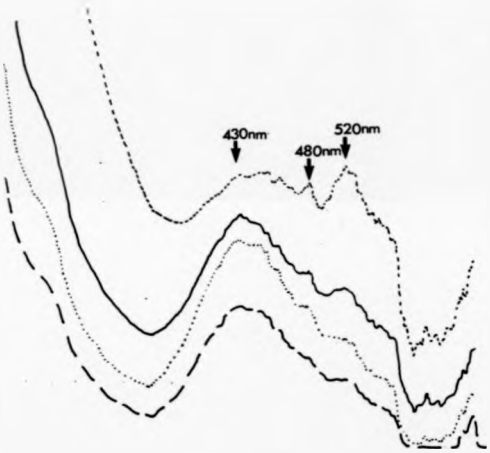


Figure 4.10 Effect of magnesium starvation on the pattern of outer membrane proteins in *Synechococcus* R2

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of cell walls isolated by sucrose gradient density centrifugation. Lane a - iron deficient medium; lane b - normal medium; lane c - magnesium deficient medium (proteins induced indicated by arrows).

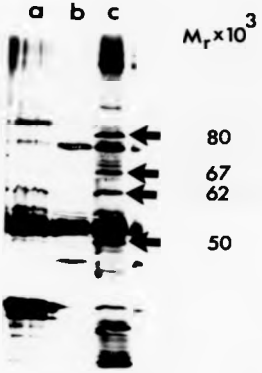
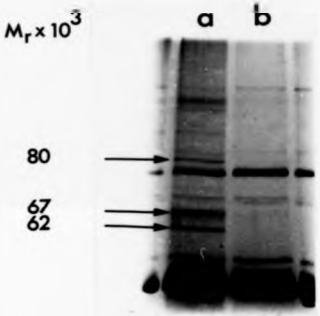


Figure 4.11 Silver-stained 10-30% (w/v) exponential gradient SDS-PAGE of Triton X-100 insolubilised cell wall fractions from *Synechococcus* R2.

Lane a - magnesium-deficient medium (polypeptides induced indicated by arrows), lane b - normal medium.



starvation. The induction of a polypeptide of approx. 50,000 molecular weight is interesting, in that this polypeptide appears to be induced under various nutrient limitations (see section 4.3.2).

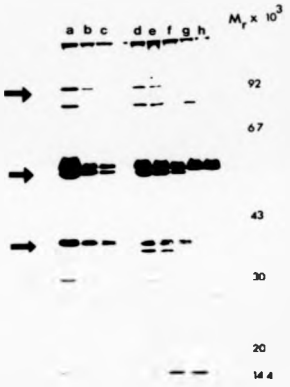
The absorption spectrum of isolated cell walls was markedly different in magnesium-deficient cells, with absorption maxima at 430 nm, 458 nm, 462 nm and 480 nm and a very dramatic increase in absorption at 520 nm. Calcium and iron-deficient cell wall fractions from *Synechococcus* R2, by contrast showed absorption spectra similar to normal growth conditions (see Fig. 4.9).

d) Iron limitation

Growth of *Synechococcus* R2 in iron-limiting or chelator deficient medium (see Section 2.4) caused significant changes in the profile of outer membrane proteins. Polypeptides observed to be induced under conditions of iron limitation had molecular weights of 92,000, 35,000 and approx. 48-50,000. Several minor polypeptides were also induced in the higher molecular weight range (see Fig. 4.12). Addition of the chelator ethylenediaminedihydroxyphenyl acetic acid (EDDA), 0.004%, to iron replete medium simulated the conditions of iron deprivation. However, the growth rate of *Synechococcus* R2 under the two conditions varied drastically (see Fig. 3.1). *Synechococcus* R2 grown in Allen's medium + EDDA had a growth rate similar to normal medium, in chelator-deficient medium the growth rate was markedly reduced. This might suggest that it is the availability of iron which causes induction of the various outer membrane proteins, and not changes in growth rate. The variation in growth rate can be explained by suggesting normal medium + EDDA restricts the availability of iron, which then derepresses specific

Figure 4.12 Effect of iron limitation on the pattern of outer membrane proteins in *Synechococcus* R2

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of consecutive sucrose density gradient fractions of isolated cell walls from *Synechococcus* R2 grown in medium containing: lanes a-c, 0.2 μM iron; lanes d-f, 2 μM iron; lanes g, h, 20 μM iron (normal medium). Arrows indicate induced polypeptides.



high-affinity iron- repressible outer membrane protein receptors - which bind iron loaded siderophores (section 4.1.2), whose synthesis is also derepressed - but once induced, the availability of iron in the medium is no longer restricting growth of the organism. In a similar manner, iron limitation induced by growing *Synechococcus* R2 in chelator-deficient medium also switches on the high affinity iron uptake systems, but here, since iron is actually limiting in the medium, the growth rate is reduced. However, caution should be employed when interpreting this data. Lodge *et al.* (1986) showed that siderophore (enterobactin) production in *Klebsiella pneumoniae* was greater at fast or slow growth rates in continuous culture than in logarithmic - or stationary - phase batch culture. The iron regulated outer membrane proteins (IRMP) and enterobactin were induced at the fast growth rate in continuous culture, but at the slow growth rate although the IRMP were barely visible, a significiant level of enterobactin was still produced. These results suggest that the growth rate can influence the induction of the high-affinity iron-uptake system of *K. pneumoniae*. Similar experiments in chemostat culture remain to be performed with *Synechococcus* R2. Whether the phenotypic variability of the outer membrane protein pattern under other nutrient limitations, is also dependent on growth rate, can also be investigated in continuous culture. It would then be possible to study the relationship between the extracellular concentration of the growth-limiting nutrient and the outer membrane protein profile, by assessing the effect of the culture dilution rate on the protein composition of the membrane.

The induction of the IRMP was observed in both chelator-deficient medium, and medium containing 0.2-2 μM $FeSO_4.7H_2O$. However, cells

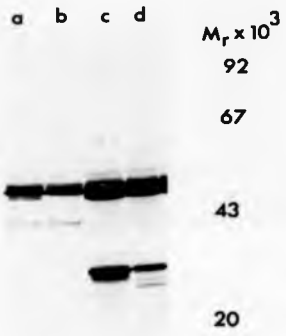
switched from an iron-replete medium to a chelator-deficient medium, and grown in the latter medium for 3-4 days, showed a less significant induction of these proteins (see Fig. 4.13). This was perhaps a reflection of the iron stores within the cell. *E. coli* is able to store iron intracellularly during periods of iron replation and use it during periods of iron stress. In this organism it has been suggested that once the intracellular iron falls below a specific level, synthesis of iron transport components becomes derepressed, and in iron replete cells the stored iron becomes accessible during the induction sequence, increasing the concentration of free intracellular iron and therefore retarding induction kinetics (Klebba *et al.*, 1982). A similar situation may be occurring in *Synechococcus* R2. These observations suggest against the idea that de-repression depends on the organisms ability to sense the extracellular iron concentration independant of the stored or free intracellular concentration.

The ability of chelators such as EDDA to cause induction of the IRMP allows an analysis of the induction process without affecting the growth rate. It is interesting that variation in expression of the IRMP in *E. coli* was noted, dependent on the chelator used to restrict the availability of iron (Chart *et al.*, 1986). They observed marked qualitative or quantitative differences in the IRMP expressed in the presence of α,α-dipyridyl, EDDA or desferal.

In order to correlate the appearance of the IRMP with siderophore production, siderophore activity was investigated under conditions of iron-limitation. However, using a universal chemical assay (see Section 4.2.5) no siderophore activity was detected in any of the cyanobacterial

Figure 4.13 Altered induction of *Synechococcus* R2 IRMP

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of the pattern of *Synechococcus* R2 outer membrane proteins after growth of the organism in: i) iron-replete medium for 2-3 days followed by 3-4 days growth in chelator-deficient medium (lane a); ii) normal medium (lane b); iii) chelator-deficient medium alone (lanes c,d).



strains examined, including *Synechococcus* sp. PCC7002, for which siderophore activity has been shown (see Boyer *et al.*, 1987). The general applicability of this method might then be questioned. Recently, the presence of an hydroxamate siderophore was demonstrated in *Synechococcus* R2 (D. Laudenbach, personal communication), using an alternative detection system. Further work may be directed towards structural analysis of the siderophore, investigation of the level of extracellular iron which induces siderophore biosynthesis, and identification of the outer membrane receptor involved in binding the iron-loaded siderophore.

4.3.2 Use of antibodies to investigate the function and export of *Synechococcus* R2 outer membrane proteins

Western blots of the IRMP showed that these proteins cross-reacted with a polyclonal antiserum (see Section 4.2.2) raised against *Synechococcus* R2 cell walls, isolated from cells grown in normal growth medium (see Fig. 4.3). This antiserum also cross-reacted against a 50,000 molecular weight polypeptide shown to be induced under low sucrose concentrations. Perhaps this polypeptide is multifunctional, or induced under 'general stress' conditions. The presence of antibody to these 'stress response' proteins, suggests that such proteins are present in very small quantities under normal growth conditions, or that they are highly cross-reactive. Attempts to raise antibodies solely against the approx. 50,000 molecular weight polypeptide, purified by preparative SDS-PAGE, were unsuccessful. The possibility that antibodies to the IRMP might interfere with iron uptake and inhibit bacterial growth remains to be investigated. This would have important implications in bacterial pathogenicity studies.

Western blots using a biotin-streptavidin detection system (Section 4.2.3) required up to 5 times more primary antibody than those using a system based on the use of peroxidase conjugated goat anti-rabbit IgG as secondary antibody. It was noteworthy that using this latter detection system, higher levels of non-specific cross-reactivity were observed when washing filters with 0.05% Tween, than using Triton X-100.

Recently, antibody against CPVI-4 - one of the photosystem II enriched chlorophyll proteins of *Synechococcus* R2, which is particularly abundant in iron-deficient cells (Pakrasi *et al.*, 1985) - was used to identify an iron-regulated membrane protein gene in *Synechococcus* R2 (Reddy *et al.*, 1987). The antibody against CPVI-4 recognised three proteins of 36,000, 35,000 and 34,000 molecular weight in low iron grown membranes. Utilising the expression vector λgt11 a gene encoding the 36,000 polypeptide was identified using antibody as a probe. This protein was shown to be present in the cytoplasmic membrane.

Using affinity purified antibody against the 36,000 molecular weight polypeptide (a kind gift of H. Reithman) we observed cross-reaction with the approx. 35,000 iron-regulated outer membrane protein, together with some cross-reaction against the 50,000-52,000 outer membrane proteins (see Fig. 4.14). Antibody (a kind gift of G. Bullerjahn) against a carotenoid binding protein of the cytoplasmic membrane of *Synechocystis* sp. PCC6714 (Bullerjahn and Sherman, 1986) also showed some cross reactivity with the 35,000 and 50-52,000 outer membrane proteins of normal and iron-starved *Synechococcus* R2. In addition, antibody (a gift of H. Reithman) purified against a 42,000 molecular weight carotenoid-associated thylakoid protein from the cyanobacterium *Synechococcus* R2

(Masamoto *et al.*, 1987) showed slight cross-reactivity with *Synechococcus* R2 outer membrane proteins, as well as cross-reactivity with approx. 40,000 and 42,000 molecular weight polypeptides of the *Synechocystis* sp. PCC6714 cell wall (see Fig. 4.15). The synthesis of the 42,000 molecular weight polypeptide was induced under high light intensities, but was absent or much decreased in iron-stressed cells (Masamoto *et al.*, 1987). The presence of carotenoids in the photosynthetic membranes and cell walls of *Synechococcus* R2 is likely to be physiologically important in their protection against photo-oxidative damage. It is possible that a family of such carotenoid-associated proteins exist, which are immunochemically related. Immunogold labelling of frozen cell sections using affinity purified antibodies will help clarify the location and relatedness of these membrane proteins.

The gene encoding the 36,000 polypeptide has been sequenced, but does not appear to possess a signal sequence (K. J. Reddy, personal communication). However, this does not exclude the possibility of the protein being present in both the cytoplasmic or outer membrane, especially since a signal sequence is not an absolute requirement for export (see Pugsley and Schwartz, 1985).

A *Synechococcus* R2 mutant lacking this 36,000 molecular weight iron-regulated protein, constructed by introducing Tn5 into the coding sequence of the gene, showed that this protein was essential for growth in iron-deficient medium (G. Bullerjahn, personal communication). This would be consistent with its location in the cell wall, and a function involved in iron uptake.

Figure 4.14 Western blot using affinity-purified antibody (1:5000dilution) against a 36,000 molecular weight IRMP from *Synechococcus* R2, and peroxidase conjugated goat anti-rabbit IgG (1:10,000 dilution) as secondary antibody

Lanes a,b - *Synechococcus* R2 OMP, chelator-deficient medium; lane c - *Synechococcus* R2 OMP, normal medium.

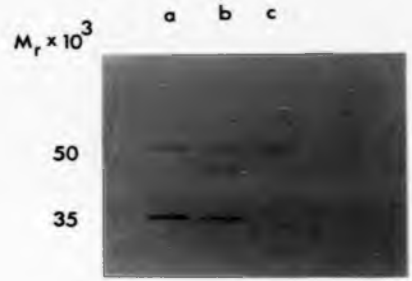
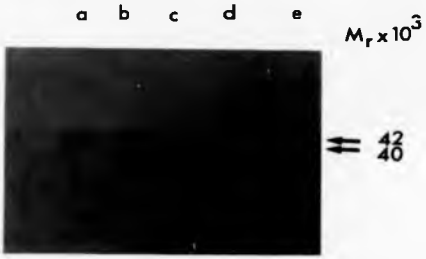


Figure 4.15 Western blot using antibody (1:1000 dilution) purified against a 42,000 molecular weight carotenoid-associated thylakoid protein from *Synechococcus* R2

Peroxidase conjugated goat anti-rabbit IgG (1:10,000 dilution) was used as secondary antibody. Lane a - *Synechococcus* R2 OMP, chelator-deficient medium; lane b - *Synechococcus* R2 OMP, normal medium; lanes c,d - *Synechococcus* sp. PCC7002 OMP, normal medium; lane e - *Synechocystis* sp. PCC6714 OMP, normal medium.



Antibodies raised against *Synechococcus* R2 cell walls were used to analyse the export of the outer membrane proteins. Soluble, thylakoid membrane, cytoplasmic membrane and cell wall protein fractions of *Synechococcus* R2 were separated by SDS-PAGE, transferred to nitrocellulose, and the Western blots screened using antisera to *Synechococcus* R2 cell walls. Cross reaction was specific to the cell wall fraction of *Synechococcus* R2, and no reaction against any cytoplasmically located unprocessed form of the outer membrane proteins was observed (see Fig. 4.16). This may be a result of the instability of the outer membrane proteins in the cytoplasm, or that they are not immunogenic in the unprocessed form (assuming that these proteins possess a leader sequence). Alternatively, the quantity of outer membrane protein precursor in the cytoplasm may be undetectable.

4.3.3 Screening of a *Synechococcus* R2 λgt11 library

Using a genomic library of *Synechococcus* R2 in λgt11 (a kind gift of K. J. Reddy) 20-30 positive plaques were identified using the polyclonal antisera to *Synechococcus* R2 cell walls (which had been preabsorbed against a bound bacterial lysate - see Section 4.2.4), after autoradiography using ^{125}I-labelled secondary antibody (see Fig. 4.17). This method produces fusion proteins synthesised in the λgt11 recombinant phage which can be recognised by antibody screening. These positive plaques await further analysis, but it should be possible to clone genes encoding the major outer membrane proteins of *Synechococcus* R2, and the IRMP.

Figure 4.16 Use of *Synechococcus* R2 cell wall antibody to study the export of *Synechococcus* R2 outer membrane proteins

a) Western blot using the Amersham biotin-streptavidin detection system. Lane a - cell wall fraction, chelator-deficient medium; lane b - cell wall fraction, normal medium; lane c - cytoplasmic membrane fraction; lane d - thylakoid membrane fraction; lane e - soluble cell protein.

b) Silver stained 10-30% (w/v) exponential SDS-PAGE. Lanes 1,12 molecular weight markers; lanes 2,3 - soluble cell protein; lanes 4, 5 - thylakoid membrane fraction; lanes 6,7 cytoplasmic membrane fraction; lanes 8,9 - cell wall fraction; lane 10 - Triton-insoluble cell wall fraction, normal medium; lane 11 - Triton-insoluble cell wall fraction, chelator-deficient medium. Cellular fractions. Lanes 2-9 were isolated using the method of Omata and Ogawa (1986).

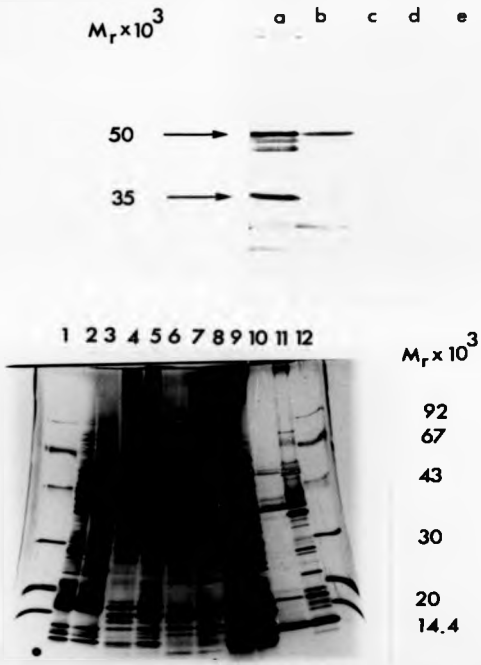
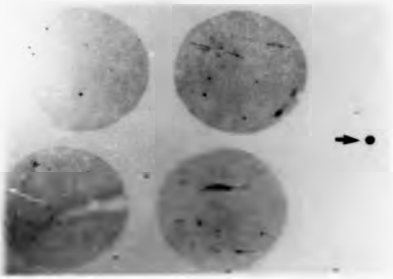


Figure 4.17 Autoradiographic signals obtained with *Synechococcus* R2 cell wall antibody (1:1000 dilution) on *Synechococcus* R2 λgt11 library using ^{125}I-labelled secondary antibody (10^3-10^4 plaques per filter).

Arrow indicates positive control - *Synechococcus* R2 cell wall fraction approx. 0.1 μg protein, spotted onto a filter.



4.3.4 Isolation and characterization of a cell wall fraction from *Nostoc* sp. MAC

A carotenoid-containing membrane fraction which has been tentatively assigned as cell wall, was isolated from *Nostoc* sp. MAC using the method described in Section 4.2.2. This orange-brown fraction occurred at sucrose densities of 60-65% sucrose, considerably less dense than the cell wall preparation from *Synechococcus* R2 (see Fig. 4.18). Extraction with Triton X-100 showed this isolated sucrose gradient fraction to be only partially insoluble in this detergent since a very unstable pellet was produced. However, a more stable Triton X-100 insoluble fraction was isolated from a whole cell lysate of *Nostoc* sp. MAC (see Section 4.2.2), which produced a brown/deep brick red pellet after centrifugation. This fraction contained three polypeptides between 56,000 and 58,000 molecular weight (see Fig. 4.19). The size of these polypeptides, which we propose comprise the major outer membrane proteins of *Nostoc* sp. MAC, are clearly different from the extracellular proteins of this organism, described in Chapter 3.

Absorption spectra of isolated cell walls, showed maxima at 440 nm, 490 nm and 525 nm - indicative of carotenoids - whereas there was no contamination with chlorophyll or phycobiliproteins (see Fig. 4.20). In contrast, the absorption spectrum of isolated cell walls from *Nostoc* sp. MAC grown in chelator-deficient medium (as compared to normal growth medium, see above) lacked an absorption maxima at 440 nm.

The major outer membrane proteins of *Nostoc* sp. MAC showed some variability in their electrophoretic mobility under different solubilization temperatures. The 58,000 molecular weight polypeptide

Figure 4.18

a) Cell wall fractions from *Nostoc* sp. MAC (arrowed) and *Synechococcus* M2, isolated by sucrose-density gradient centrifugation 50-85% (w/v)

b) Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of fractions from the *Nostoc* sp. MAC cell wall preparation, shown in a); lanes 1 & 11 molecular weight markers.

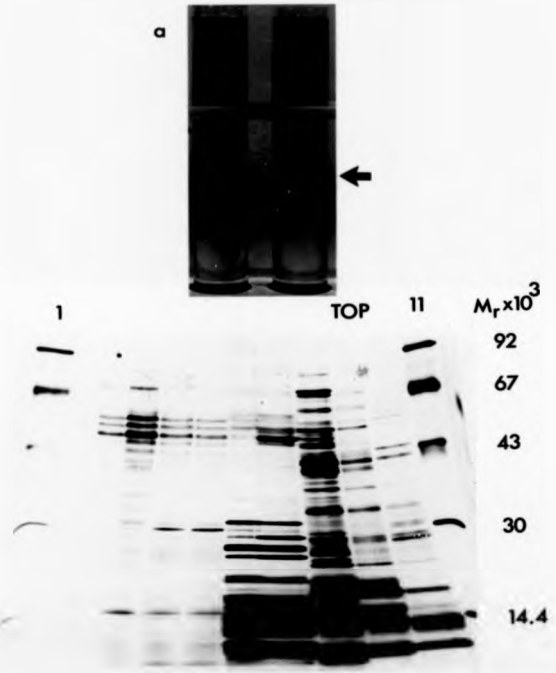


Figure 4.19 Silver stained 10-30% (w/v) exponential gradient SDS-PAGE of purified Triton X-100 insoluble cell wall fractions from Synechococcus R2. lanes a, b. Nostoc sp. MAC - lanes c, d

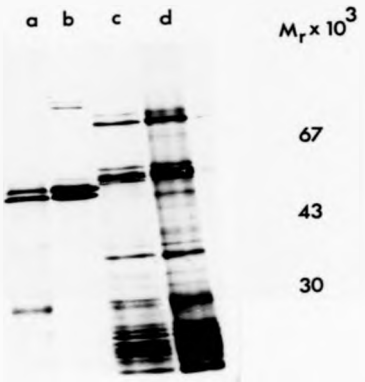
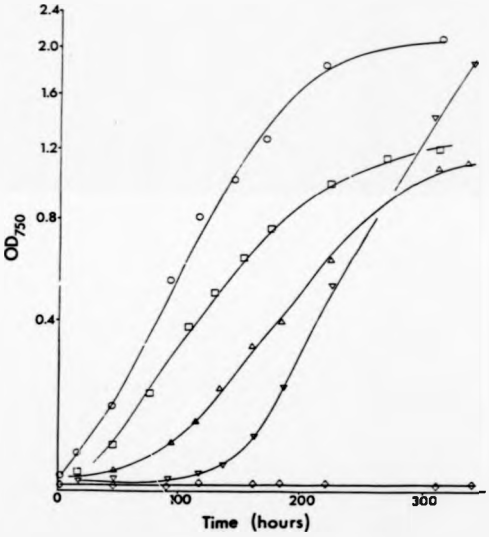


Figure 4.20 Absorption Spectra of isolated cell wall fractions from Nostoc sp. MAC grown in i) normal medium ——, and ii) chelator-deficient medium - - -.

Figure 4.21 The effect of 0.1% casamino acids and 10 mM glucose on the growth of *Nostoc* sp. MAC in the light and dark

Graph of growth (OD_{750}) against time (hours). Growth of *Nostoc* sp. MAC in i) normal medium, light □----□; ii) normal medium + 0.1% casamino acids, light (inoculated from dark grown culture see iv) ○----○; iii) normal medium + 0.1% casamino acids + 10 mM glucose, light (inoculated from dark grown culture, see iv) ▽----▽; iv) normal medium + 0.1% casamino acids + 10 mM glucose, dark △----△; v) normal medium + 0.1% casamino acids, dark ◇----◇.



was completely solubilized by heating at 100°C, but only partially soluble at 65°C, and below this temperature was absent. The mobility of the 56,000 polypeptide was not altered by variation in solubilization temperature (see Fig. 4.4).

Growth of *Nostoc* sp. MAC in chelator-deficient medium or normal medium + EDDA caused no change in the outer membrane protein profile as assessed by SDS-PAGE. The growth rate did not change significantly under the two conditions (see Fig. 3.2), and this may suggest that the cells were not completely iron stressed. Repeated subculture of *Nostoc* sp. MAC into iron-limiting medium did not affect the growth rate appreciably. However, *Nostoc* sp. MAC has been shown to possess a periplasmic iron-binding protein (Section 3.3.4) and this may constitute an alternative mechanism for iron uptake in this organism. The slower growth rate of this organism compared to *Synechococcus* R2, for example, may allow *Nostoc* sp. MAC to achieve its iron requirements by a constitutive low affinity system, which uses such a periplasmic-binding protein. Alternatively, *Nostoc* sp. MAC may possess an efficient iron-storage molecule, which can be utilized and distributed along the filament in times of iron limitation. This may avoid the requirement for a specific high-affinity iron uptake system. Considerable iron storage capability has been demonstrated in other cyanobacteria, by Mossbauer spectroscopy (Evans *et al.*, 1977).

Since *Nostoc* sp. MAC can grow heterotrophically in the dark, we attempted to investigate the utilization of casamino acids and glucose by this organism, and analyse any changes in the outer membrane protein profile during growth of *Nostoc* sp. MAC under such conditions. This

might result in the induction of specific transport systems for these nutrients. *Nostoc* sp. MAC could not utilise 0.1% casamino acids as the sole carbon source in the dark, but supplementing the medium with 10 mM glucose allowed growth, though a long lag phase was observed. Addition of 0.1% casamino acids to normal growth medium in the light, considerably increased the growth rate, suggesting utilization of this C and N source (see Fig. 4.21 Bottomley and Van Baalen, 1978). However, variability in the consistency and repeatability of membrane quality under these conditions meant that no significant conclusions could be drawn from this analysis.

4.3.5 Comparative studies of outer membrane proteins from other cyanobacteria

Cell wall fractions containing outer membrane plus peptidoglycan were isolated from various unicellular cyanobacteria: *Synechococcus* sp. PCC7002 and *Synechocystis* sp. PCC6308, both capable of growth in chelator-deficient medium (see Boyer *et al.*, 1987), the former a marine species and the latter a freshwater species; and *Synechocystis* sp. PCC6803, *Synechocystis* sp. PCC6714, capable of photoheterotrophic growth. A cell wall fraction was also isolated from the prochlorophyte *Prochlorothrix hollandica* (a gift of L. Sherman).

The cell wall fraction from *Synechococcus* sp PCC7002 (absorption maxima at 460 nm, 515 nm) and *Prochlorothrix hollandica* (absorption maxima at 458 nm, 480 nm and 515 nm) was a yellow band, occurring at sucrose densities of 75-80% (w/v) (1.28 g cm^{-3}), similar to *Synechococcus* R2. In contrast, the isolated cell wall fraction of *Synechocystis* PCC6308 (absorption maxima at 450 nm and 520 nm, see Fig. 4.26), *Synechocystis*

PCC6803 (absorption maxima at 450 nm and 520 nm, see Fig. 4.26) and *Synechocystis* PCC6714 (absorption maxima at 460 nm) corresponded to an orange band, occurring at sucrose densities of 80-85% (w/v) (1.31 g cm^{-3}) (see Fig. 4.22). No contamination with chlorophyll or phycobiliproteins was observed. Cyanobacterial cell wall preparations appear to have significantly higher buoyant densities than those of the enteric bacteria (1.28-1.31 g cm^{-3} as compared with 1.22 to 1.25 g cm^{-3}, Resch and Gibson, 1983).

SDS-PAGE analysis of isolated cell wall fractions (see Figs. 4.23 and 4.27) showed that the major outer membrane proteins from these cyanobacteria were of considerably higher molecular weight than the 37,000-41,000 molecular weight polypeptides which comprise the major outer membrane proteins in Enterobacteriaceae species (see Table 4.1).

Western blotting using antisera to *Synechococcus* R2 cell walls, showed some cross-reactivity against the major outer membrane proteins from *Synechocystis* sp. PCC6308, *Synechocystis* sp. PCC6803, *Synechocystis* sp PCC6714 and *Prochlorothrix hollandica* (see Figs. 4.3 and 4.24) suggesting that these proteins are immunogenically related. No cross reactivity was observed with *Synechococcus* sp. PCC7002, a marine species. Whether this is significant will require further analysis of outer membrane proteins from both marine and freshwater cyanobacteria. Considerable antigenic cross-reactivity of outer membrane proteins between most *E. coli* serotypes has been reported (Hofstra and Dankert, 1979).

Apart from the 34,000 molecular weight polypeptide from *Synechocystis* sp. PCC6308 which was observed to be solubilized at room temperature in SDS, the major outer membrane proteins of *Synechocystis* sp. PCC6308, and *Synechocystis* sp. PCC6803 were solubilised with SDS only by heating at high temperature (see Fig. 4.4). This feature appears to be a common property of cyanobacterial outer membrane proteins.

Growth of *Synechocystis* sp. PCC6308 in chelator-deficient medium caused a significant change in the outer membrane protein profile of this organism. Polypeptides of 52,000 and 36,000 molecular weight were markedly induced under these conditions, and to a lesser extent polypeptides of approx. 70,000 and 67,000 molecular weight (see Fig. 4.27). The 52,000 molecular weight polypeptide was solubilised in SDS only at high temperature. This phenomenon appears very similar to that observed with *Synechococcus* R2. Similar growth of *Synechocystis* sp. PCC6803 in chelator-deficient medium, showed no significant change in the outer membrane protein profile.

Growth of *Synechocystis* sp. PCC6308 and *Synechocystis* sp. PCC6803 in normal medium + 0.1% casamino acids in the light, caused no significant change in the outer membrane protein profile (see Fig. 4.27). This would be consistant with the non-specific passage of amino acids through the major porins of the outer membrane. Further work in this area should be aimed at analysis of specific periplasmic proteins, for nutrient binding, and specific membrane-bound transport systems of the cytoplasmic membrane.

Figure 4.22 Cell wall fractions (arrowed) from various cyanobacteria and a prochlorophyte, isolated by sucrose density gradient centrifugation 50-85% (w/w)

Organisms were grown in normal medium, unless otherwise stated.
a) *Synechocystis* sp. PCC6714; b) *Prochlorothrix hollandica* ACC-15-2; c) *Synechococcus* R2; d) *Synechococcus* R2 chelator-deficient medium; e) *Synechococcus* sp. PCC7002; f) *Synechococcus* R2.

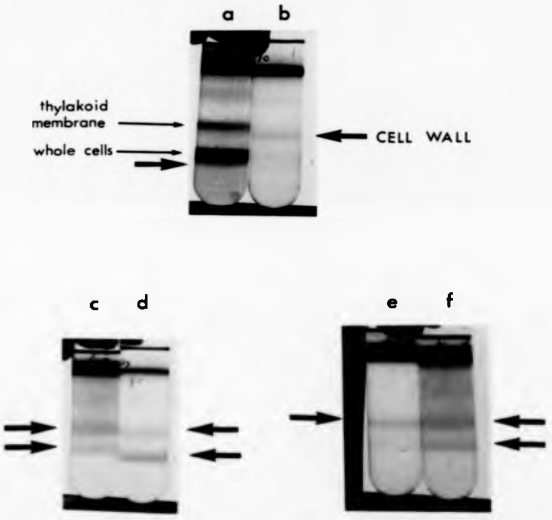


Figure 4.23 Coomassie-stained 10-20% linear gradient SDS-PAGE of outer membrane proteins from various cyanobacteria, and a prochlorophyte

Organisms were grown in normal medium unless otherwise stated.
Lanes, a, g, h - *Synechococcus* R2 iron-deficient medium; lane b - *Synechococcus* R2; lanes c, d *Synechococcus* sp. PCC7002; lane e - *Synechocystis* sp. PCC6714; lane f - *Prochlorothrix hollandica* ACC-15-2.

Figure 4.24 Western blot using antibody (1:1000 dilution) raised against a cell wall fraction from *Synechococcus* R2

Peroxidase conjugated goat anti-rabbit IgG (1:10,000 dilution) was used as secondary antibody.

Lane a - *Prochlorothrix hollandica* OMP, normal medium; lanes b, c *Synechococcus* R2 OMP, iron-deficient medium; lane d - *Synechococcus* R2 OMP, normal medium; lanes e, f - *Synechococcus* sp. PCC7002 OMP normal medium; lane g - *Synechocystis* sp. PCC6714 OMP, normal medium.

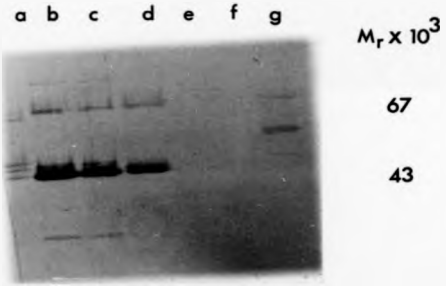


Figure 4.25

a) Cell wall fraction from *Synechocystis* sp. PCC 6803 (arrowed) isolated by sucrose density gradient centrifugation (50-85% w/v).

b) Silver-stained 10-30% (w/v) exponential gradient SDS-PAGE of fractions from the sucrose gradient shown in a).

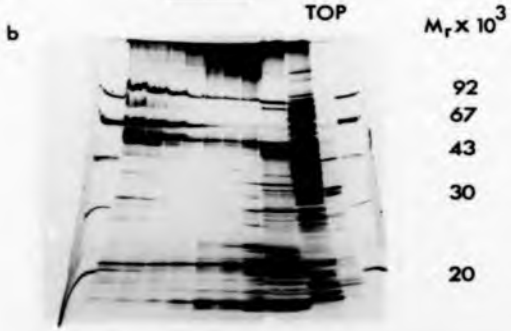


Figure 4.26 Absorption spectra of isolated cell wall fractions

From a) *Synechocystis* sp. PCC6803, and b) *Synechocystis* sp. PCC6308 grown in i) normal medium ——, ii) chelator-deficient medium - - - -.

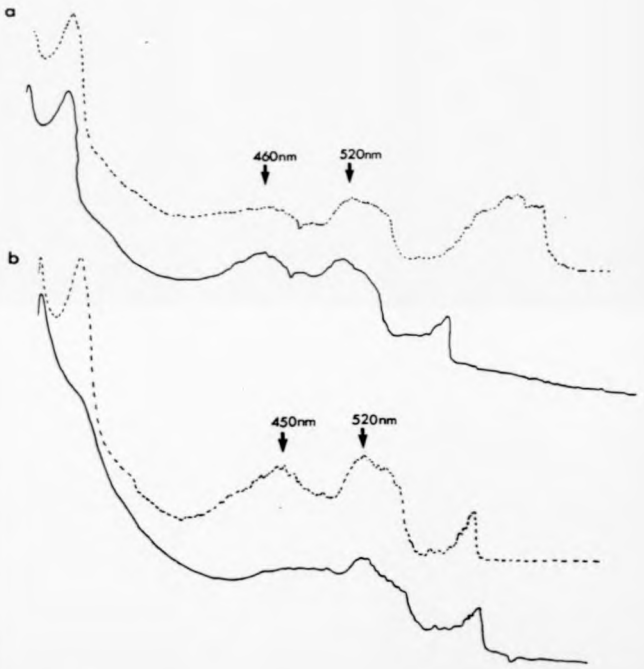


Figure 4.22 Triton X-100 insoluble outer membrane proteins of *Synechocystis* sp. PCC6803 and *Synechocystis* sp. PCC6308

Silver stained 10-30% (w/v) exponential gradient SDS-PAGE. Lanes a, b - *Synechocystis* sp. PCC803, normal medium; lane c - *Synechocystis* sp. PCC6803, normal medium + 0.1% casamino acids, light; lane d - *Synechocystis* sp. PCC6803 chelator deficient medium; lane e - *Synechocystis* sp. PCC6308 normal medium; lane f - *Synechocystis* sp. PCC6308 normal medium + 0.1% casamino acids, light; lane g - *Synechocystis* sp. PCC6308 chelator deficient medium (arrows indicate polypeptides induced under these conditions).

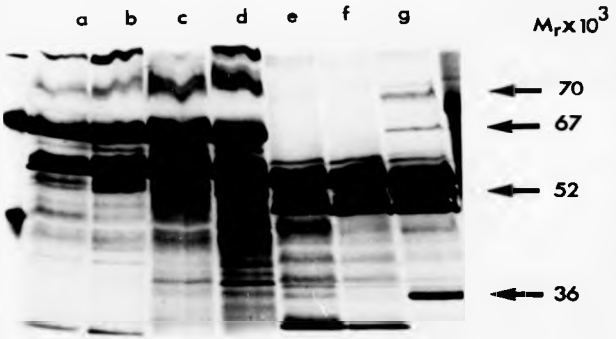


Table 4.1 Major outer membrane proteins of various cyanobacteria and a prochlorophyte

Cyanobacteria/Prochlorophyte	Major outer membrane proteins (molecular weight)
Synechococcus R2	52,000, 78,000
Synechococcus sp*	52,000, 78,000
Synechococcus sp. PCC7002	58,000, 67,000
Synechocystis sp. PCC6308	34,000, 50,000, 54,000
Synechocystis sp. PCC6803	55,000, 60,000, 67,000
Synechocystis sp. PCC6714	61,000, 66,000, 67,000
Nostoc sp. MAC	58-60,000, 70,000
Prochlorothrix hollandica	55,000, 60,000

*This organism is a strain of *Anacystis nidulans* (see Chapter 6)

4.3.6 Southern-blotting analysis of cyanobacterial genomic DNA using the *E. coli* ompC gene as probe

OmpC is one of the major outer membrane proteins of *E. coli*, and shares extensive DNA homology between the OmpF gene of *E. coli*. These two proteins resemble each other with respect to the trimeric structure that is resistant to SDS and pore functions for small hydrophilic molecules.

To assess the homology, at the DNA level, between *E. coli* and cyanobacterial outer membrane proteins, and hence the prospective cloning of a cyanobacterial outer membrane protein gene, the OmpC gene of *E. coli* was used to probe genomic DNA from two cyanobacteria. pMAN006 (a kind gift of C. Higgins) contains a 2.7 kb *Hin*dIII DNA fragment encoding the OmpC polypeptide (Matsuyama *et al.*, 1984) and this, together with a 550 bp internal *Pvu*II-*Bgl*II fragment of Omp C were used as probes. Total genomic DNA was isolated from *Nostoc* sp. MAC and *Synechococcus* R2 and cut with *Hin*dIII and *Eco*RI. Southern blotting analysis showed no significant homology to *omp*C, at stringencies ranging from 20-45% base-pair mismatch. This latter figure was calculated from: $Tm = 69.3 + 0.41 \times (G+C)\% - \frac{650}{L}$ were L is the length of the probe in nucleotides. Tm is decreased by $1^{\circ}C$ for every 1% base pair mismatch (Maniatis *et al.*, 1982). *Synechococcus* R2 has a (G+C)% of 55%; this value has not been calculated for *Nostoc* sp. MAC, but other species of *Nostoc* possess a (G+C)% of approx. 40%. Hybridisation experiments were performed at a) $65^{\circ}C$ in 5 x SSPE and filters washed for 1 hr, i) at room temperature in 2 x SSPE, ii) at $65^{\circ}C$ in 2 x SSPE; b) $55^{\circ}C$ in 5 x SSPE and filters washed for 1 hr at i) room temperature in 2 x SSPE, ii) $50^{\circ}C$ in 2 x SSPE, iii) $55^{\circ}C$ in 2 x SSPE and iv) $55^{\circ}C$ in 1 x SSPE. *E. coli* strain DH1 DNA hybridised strongly to the *omp*C DNA probe. The lack of

homology shown here, suggests that at the DNA level, cyanobacterial and *E. coli* outer membrane proteins are not closely related.

4.4 Conclusions

An analysis of cyanobacterial outer membrane proteins has shown that the extracellular polypeptides described in the previous chapter, were not simply intact components of the cell wall discarded into the culture supernatant. Moreover, outer membrane proteins from *Synechococcus* R2 appear to have important functions in nutrient uptake especially under conditions of nutrient starvation. It is possible that this organism induces specific proteins in the outer membrane as a general 'stress response', and such multifunctional polypeptides clearly require further analysis. Iron starvation particularly, caused a dramatic induction of outer membrane proteins, and this may be a widespread phenomenon among cyanobacteria.

The 'consistency and repeatability of membrane quality' (section 4.3.5) is clearly important in these investigations, and this is considerably more difficult to fulfill for the filamentous cyanobacteria. The isolation of a cell wall fraction from *Nostoc* sp. MAC is the first report of outer membrane isolation from a filamentous cyanobacteria.

Since outer membrane proteins represent 'exported' proteins, their analysis has shown how important this class of proteins are in maintaining the correct internal balance of nutrients, essential for cellular metabolism, with changing environmental stimuli. The cloning of genes encoding specific outer membrane proteins (see Chapter 8), and

subsequent mutant construction will further clarify the function of these exported proteins.

Chapter 5

Expression of Foreign Genes in Cyanobacteria

5.1 Introduction

The current state of cyanobacterial molecular biology has been discussed in general terms (see section 1.8) and therefore this section will only address the problems of foreign gene expression in cyanobacteria. Suitable gene transfer systems for both unicellular (see Shestakov and Reaston, 1987) and filamentous strains (Wolk *et al.*, 1984) are established, and hence the introduction of foreign genes into cyanobacteria has become a realistic proposition.

Our objectives were to express specific foreign genes encoding polypeptides that were considered suitable markers for cytoplasmic, periplasmic and extracellular compartments of the cell. Attention has been concentrated on the unicellular strain *Synechococcus* R2 which has a well-developed transformation system (Kuhlemeier, 1981), a high plating efficiency and appeared to lack *in vivo* restriction enzyme activity (but, see section 5.1.2). The transfer of foreign genes into *Nostoc* sp. MAC using the conjugative shuttle vector system developed by Wolk *et al* (1984) has also been attempted.

5.1.2 Factors required for the expression of a given gene into an heterologous host

It should be noted that expression of heterologous genes has been observed in cyanobacteria (see section 5.1.4). This implies, i) that problems arising from the presence of any endogenous restriction system have been overcome, ii) that the gene has a promotor region which is recognised by the host transcriptional machinary, iii) that the transcript carries a sequence able to bind host ribosomes upstream from the translation initiation site, and iv) that the organism from which

the gene originates and the host must have compatible codon usages.

Recently, *Synechococcus* R2 has been shown to possess a sequence-specific endonuclease which could be expected to restrict transformation with foreign DNA in this strain (Gallacher and Burke, 1985). This enzyme, *Ani* I, appears to recognise a sequence which contains the *dam* sequence (GATC) and is sensitive to *dam* methylation. Fortuitously, all the shuttle vectors for *Synechococcus* R2 were constructed in *dam*$^+$ strains of *E. coli*, and as such provide a means of avoiding this problem.

Nostoc sp. MAC also produces a type II restriction endonuclease. This enzyme Nsp. MACI, is an isoschizomer of Bgl II and recognises the sequence A↓GATCT. The presence of Nsp. MACI in this strain could seriously lower the efficiency of introduction of foreign DNA. It may be possible to generate mutants of this strain defective in the production of this enzyme, to afford protection of transferred DNAs containing its recognition sequence against possible degradation within the cell. The problem of host restriction with regard to the system of gene transfer developed by Wolk *et al.* (1984), and the method of conjugation used here, is discussed later in this chapter.

Only few cyanobacterial promoter sequences have so far been published (Table 5.1) and they share few common features. The *nif H,D* and nif-like *gln A* promoters are themselves similar, but are very different from the *E. coli* consensus promoter (Pribnow, 1975). The sequences in the -35 region seem quite unrelated to that assigned to the *E. coli* consensus promoter. In the -10 region the sequence TTCTA resembles more that of the *K. pneumoniae nifA* activated *nif* promoters, TTGCA than the classical

Table 5.1 Promoter sequences of cyanobacterial genes

Organism	Gene	"-35 region"	"-10 region"
Synechococcus PCC6301	rbcL-rbcS	TTCAAT	TAGGT
(*Anacystis nidulans*)		-38 to -36	-13 to -11
Synechococcus PCC7002	cpcB-cpcA	TGTTTA	TATAAT
(*Agmenellum*		-36	-13
quadruplicatum PR6)		ATGACA	TAATGT
		-35	-12
Anabaena PCC7120	nifH-nifD	CATAAC	TCTACT
		-41	-14
	glnA "nif-like"	CAAAAC	TCTACG
		-35	-13
	glnA "E. coli-like"	TTGTGC	TAATAT
		-37	-13
	rbcL-rbcS	GAATAA	TATATT
		-35	-12
	psbA1	n.d.	TAAATT
			-10
Escherichia coli	consensus	TTGACA	TATAAT
	sequence	-36 to -34	-14 to -11

(from Tandeau de Marsac and Houmard, 1987)

TATAAT Pribnow base. The other cyanobacterial genes appear to have -10 regions which resemble each other and the putative RNA polymerase-binding consensus sequence (TATAAT), but the -35 regions clearly differ.

If we assume that promoter recognition has occurred and that a transcript is being made, the next problem is that ribosomes must bind to it at a proper location in order to start translation. The step-by-step assembly of initiation complexes has, however, not yet been studied in cyanobacteria nor have the protein and cofactor requirements been defined. The only information available concerns the presence of putative Shine-Dalgarno sequences, which were found to be perfectly conserved with respect to *E. coli* 16S rRNA in the only cyanobacterial 16S rRNA sequenced to date - *Synechococcus* PCC6301 (Tomioka and Sugiura, 1983).

All cyanobacteria so far studied, seem to use the genetic code common to both other prokaryotes and the eukaryotic chloroplasts, for translating nucleotide sequences into polypeptide chains.

5.1.3 Expression of cyanobacterial genes in *E. coli*

If we consider the converse situation to that under investigation, it seems there is good agreement with the predictions one can make from the sequence data in Table 5.1, and the expression of the cyanobacterial gene in *E. coli*. The only cyanobacterial genes shown to be expressed in *E. coli* are *Anabaena* PCC7120 *glnA*, *Synechococcus* PCC7002 *cpcA,B* and *Synechococcus* PCC6301 *rbcL,S* genes. Of these genes, the -10 and -35 regions fit approximately the *E. coli* consensus sequences. The expression of cyanobacterial genes in *E. coli* is particularly important

if one is trying to clone genes by complementation of *E. coli* mutants and so it seems relevant to stress the point of the differences that there might be between cyanobacterial and *E. coli* DNA sequences susceptible to being recognised by the host RNA polymerase and/or ribosome binding site.

5.1.4 Expression of heterologous genes in cyanobacteria

The gene encoding ampicillin resistance carried by Tn 901 has been expressed in *Synechococcus* R2, although to a much lesser extent than in *E. coli*, and served to develop cloning vehicles for this organism (van den Hondel *et al.*, 1980). Cloning vectors have also been constructed which make use of *lac Z* (Buxby *et al.*, 1985; and see section 5.3.2) or lambda c1857 (Fraidberg and Seijffers, 1986) expression in cyanobacteria. In addition, Elanskaya *et al.* (1985), and Gallacher and Burka (1985) have demonstrated the expression of genes originating from Gram-positive bacteria in *Synechococcus* R2. The expression of the *lux* genes from *Vibrio harveyi* and *Vibrio fischeri* into *Anabaena* spp. has been used for construction of promoter probes for analysis of heterocyst differentiation (Schmetterer *et al.*, 1986), and this is discussed more fully in Chapter 7. Dzelzkalns *et al* (1984), showed that the expression of the chloramphenicol acetyl transferase (CAT) gene can be driven in *Synechococcus* PCC6301 by a chloroplast *psb*A gene promoter, believed to be a strong promoter in that organelle. This result underlines the possible cyanobacterial origin of chloroplast genes and paves the way for testing the ability of chloroplast gene products to function in the cyanobacterial photosynthetic apparatus.

5.1.5 Replication of *E. coli* vectors in unicellular cyanobacteria

No *E. coli* vector or broad host range plasmid has yet been found capable of autonomous replication in unicellular cyanobacteria (van den Hondel *et al.*, 1980; Kuhlemeier *et al.*, 1981; Lightfoot *et al.*, 1987). Daniell *et al.*, 1986 reported the transformation of the *E. coli* vector pBR322 into permeaplasts of *Synechococcus* PCC6301, however, Lightfoot *et al.* (1987) have been unable to isolate intact molecules of pBR322 in the same strain. Dzelzkalns and Bogorad (1986) observed that UV irradiated *Synechocystis* PCC6803 cells integrated *E. coli* plasmids into the chromosome even though they did not contain a cyanobacterial origin of replication - this integrative transformation was independent of homologous recombination. It could be that a similar integration mechanism had occurred in the experiments described by Daniell *et al.* (1986). Indeed, Delaney and Reichelt (1982) have demonstrated the integration of the conjugative plasmid R68.45 into the genome of *Synechococcus* PCC6301.

The inability of *E. coli* vectors to replicate autonomously in cyanobacteria, meant that the introduction of foreign genes into these organisms required the construction of hybrid vectors, containing either a cyanobacterial replication origin, or a piece of cyanobacterial chromosomal DNA.

5.1.6 Transformation using integratable or shuttle vectors?

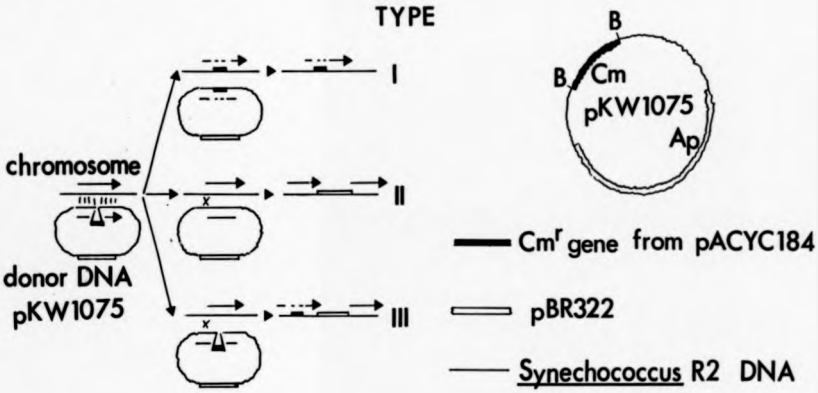
Although we have now considered the factors responsible for foreign gene expression, and have noted the requirement for construction of hybrid vectors, we still had the problem of determining the most suitable location of introduced foreign DNA in the cyanobacterial cell, i.e. was

a chromosomal, or plasmid location preferable? We took the approach in *Synechococcus* R2 of introducing genes mainly on integrative vectors, which rely on homologous recombination for their insertion into the chromosome (see below). Such introduced genes are stably maintained (S. Shestakov, personal communication), and hence this provides a useful technique for stabilising the inheritance of heterologous genes that are perhaps not so easily maintained on autonomously replicating vectors. We have, however, also introduced genes onto multicopy plasmids using the host shuttle vector systems available for *Synechococcus* R2, with the idea that this might provide information on levels of expression of gene products expressed from both a multi-chromosome organism (Herdman *et al.*, 1979; Doolittle, 1979), or a multicopy independently replicating plasmid.

The stable integration of modified chromosomal DNA was first demonstrated in *Synechococcus* R2 by Williams and Szalay (1983). They constructed chimeric DNA consisting of three elements: i) a fragment of cyanobacterial chromosomal DNA which mediates integration of the foreign DNA into the chromosome, ii) a fragment of interrupting DNA inserted within the chromosomal fragment (in this case the Cm^r gene from pACYC184), and iii) a fragment of foreign DNA flanking but not interrupting the insertional chromosomal fragment (pBR322 encoding Ap^r used here). This hybrid vector allowed autonomous replication in *E. coli*, and chromosomal integration into *Synechococcus* R2. Three types of transformants in *Synechococcus* R2 were obtained: type I resistant to Cm only; type II resistant to Ap only, and type III resistant to both antibiotics (see Fig. 5.1). Type I transformants are produced by replacement of recipient DNA by homologous donor DNA containing the

Figure 5.1 Model for integration of foreign DNA into *Synechococcus* R2

Homologous pairing between donor and recipient DNA is designated by a series of vertical dashes. To generate a type I transformant, chromosomal DNA is replaced by homologous donor DNA that contains the Cm-resistance gene. In the type II transformant the Cm-resistance is eliminated from the donor DNA instead of being transferred to the recipient. A subsequent reciprocal cross-over event (X) leads to integration of pBR322. Type III transformants can be generated by a single reciprocal cross-over. The donor DNA was pKW1075. (From Williams and Szalay, 1983).



Cm-resistance gene. These are the predominant transformants, indicating the perhaps more efficient integration of "interrupting" rather than "flanking" DNA.

Kolowsky *et al.* (1984) showed that the length of foreign DNA rather than its position in the donor molecule determined the ratio of the three types of transformants obtained. However, the most stable DNA integrate was found in type I transformants only when the foreign DNA was within the cyanobacterial "insertional" DNA. Foreign DNA linked to the ends of the cyanobacterial fragment can integrate only in type II and III configurations. Foreign DNA of 20 kb in length was found to be completely stable in type I transformants.

5.2 Materials and Methods

All molecular biological techniques and transformation procedures were performed as described in Chapter 2. Cellular fractionation procedures were described in Section 3.2.3 and protein estimation was by the Biorad method (Section 2.7.3).

5.2.1 *β*-galactosidase assays

β-galactosidase activity was assayed using i) o-nitrophenyl-*β*-D-galactopyranoside (ONPG) as described by Miller (1972), and ii) using 4-methyl umbelliferyl-*β*-D-galactoside (MUG) (see Youngman *et al.*, 1985).

i) ONPG assays

A cell lysate (50 *μ*l), a periplasmic fraction (100 *μ*l) or culture supernatant (250 *μ*l) was dispensed into Eppendorf tubes, and the volume

made up to 500 µl with Z-buffer (see Table 5.2). A fresh solution of ONPG, 4 mg ml^{-1} was made up in Z-buffer, and 100 µl added to each tube. The time was noted, and tubes were incubated at 37°C until a faint yellow colour appeared (i.e. approx. 140 min for supernatants or 40 min for sonicates). The reaction was stopped by the addition of 250 µl 1M Na_2CO_3 and the time noted. The absorbance was read at 420 nm, and data expressed as the increase in OD_{420} min^{-1} ml^{-1}. Control tubes were performed exactly as above except 100 µl Z-buffer was added instead of 100 µl ONPG.

ii) MUG assays

When MUG is hydrolysed by β-galactosidase, a methylumbelliferone is generated which is highly fluorescent under long-wavelength ultraviolet light. This allows sensitive detection of β-galactosidase activity by bacterial colonies, either visually or photographically. In contrast with colour reactions produced by bacterial colonies grown on agar plates that include X-gal, which reflect substrate hydrolysis throughout the development of the colony, MUG can detect the quantity of β-galactosidase present in bacteria at the time the substrate is applied.

Table 5.2 Constituents of Z-buffer (per litre)

8.52 g	Na_2HPO_4 anhydrous
6.24 g	$NaH_2PO_4.2H_2O$
0.75 g	KCl
0.25 g	$MgSO_4.7H_2O$
2.7 ml	β-mercaptoethanol
[pH 7.0]	Do not autoclave

MUG was applied after bacterial colonies had developed, by spraying the plate with a 10 mg ml^{-1} MUG solution prepared in DMSO. Plates were held 30 cm away from the atomizer nozzle, and a fine spray of MUG was delivered over the surface of the plate. After 5-10 min, plates were visualized under long wavelength ultraviolet light. A 2 mg ml^{-1} MUG solution prepared in DMSO was also used, for less sensitive β-galactosidase assay.

Quantitative fluorimetric assay for β-galactosidase specific activity was carried out as follows: 1 ml of a bacterial culture of a known optical density was pelleted by spinning for 1 min in an Eppendorf centrifuge, and the cells resuspended in AB buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl). If moderately low activity was expected cells were resuspended in 500 µl AB buffer, but for very low expected activity cells were resuspended in 100 µl AB buffer. If very high activity was expected, cells were resuspended in 500 µl AB and diluted 10-20x. 50 µl of the resuspended sample was transferred to an Eppendorf tube, and 10 µl of 0.4 mg ml^{-1} MUG in DMSO added, to start the reaction. The time was noted, and the tubes incubated at room temperature for approx. 100 min. The reaction was "stopped" by removing 50 µl and diluting into 2.45 ml AB buffer. Samples were read in a Perkin Elmer LS-5 luminescence spectrophotometer. For a negative control, 50 µl AB buffer was handled as if it were a sample. The optimal excitation wavelength was 366 nm, and peak emission was at 445 nm. The fluorimeter was zeroed against the blank, and then a reference sample of 4-methylumbelliferone (Sigma) at a concentration of 400 nM was prepared. The fluorimeter reading was adjusted to give a reading of 400, which meant that the fluorimeter display gave the actual concentration of hydrolysed

substrate in nM units. The specific activity was defined as one picomole of MUG hydrolysed per ml of culture sample per min, normalised for culture density.

$$\text{units} = \frac{\text{(fluorimeter display) (1000) (2.5/1000) (60/50)}}{\text{(ml of culture per assay) (duration of assay in min) } (OD_{750})}$$

5.2.2 Alkaline phosphatase assay

This assay was performed as described by Brickman and Beckwith (1975). 100 µl of lysate, periplasmic fraction or supernatant was added to 900 µl 1 M Tris (pH 8.0) in Eppendorf tubes. 100 µl of Allens medium, or 100 µl Luria broth was used as a blank. A 4 mg ml^{-1} solution of p-nitrophenyl phosphate (Sigma) was prepared in 1 M Tris pH 8.0, and 100 µl added to each tube. The time was noted, and the tubes then incubated at 37°C until a faint yellow colour appeared. The reaction was stopped by the addition of 100 µl K_2HPO_4 (filter-sterile) and the time noted. The absorbance at 420 nm was read, and the data expressed as the increase in OD_{420} min^{-1} ml^{-1}.

5.2.3 Detection of pectate lyase activity

i) Pectate lyase plate assay

This method was modified from Andro *et al.* (1984). Following incubation at 37°C, plates (see section 2.4), containing patched bacterial colonies, were flooded with 1% (w/v) cetrimethyl ammonium bromide (CTAB), and agitated gently for 2-4 hr. The CTAB precipitated the non-degraded polygalacturonic acid. Pectate lyase positive colonies gave a clear halo upon an opaque white background.

ii) Pectate lyase spectrophotometric assay

The assay was performed as described by Starr *et al* (1977) except that the reaction volume was approx. 0.8 ml. The breakdown of poly-galacturonate to yield unsaturated digalacturonate was monitored by recording the increase in absorbance at 235 nm.

To a cuvette, the following pre-heated (37°C) reagents were added: 0.34 ml reaction buffer (76.8 ml H_2O, 23.1 ml 1 M Tris, pH 8.5, 0.078 ml 1 M $CaCl_2$ per 100 ml), 0.32 ml 5.75 mg ml^{-1} polygalacturonic acid - PGA - (prepared by suspending appropriate amounts of PGA in water and slowly adding NaOH, until the pH was approx. 5.5, and no particulate PGA was visible), and 133 µl H_2O or 113 µl H_2O + 20 µl enzyme. The cuvette was mixed by inversion, and inserted into a temperature-controlled cuvette holder, set at 37°C. The absorbance increase at 235 nm was recorded, for sufficient time to allow determination of the slope. The data was expressed as the increase in OD_{235} min^{-1} ml^{-1} mg $protein^{-1}$.

5.2.4 Conjugation between *E. coli* and *Nostoc* sp. MAC

The *E. coli* strain S17.1 (Simon *et al*., 1983) which contains a derivative of RP4 (RP4-2-Tc :: Mu-Km :: Tn7) integrated into, and immobilised in, its chromosomal DNA, was routinely used as the donor strain. 1 ml of an overnight culture of *E. coli* was mixed with 1 ml of a late log phase culture of *Nostoc* sp. MAC, and filtered onto 0.45 µM Millipore filters. The filter was placed on an Allen's medium plate at 30°C for 2 days, and then the cells were resuspended in Allen's Medium and plated on Allens Medium plates containing the appropriate concentration of antibiotic. Putative transconjugants appeared after one week.

5.3 Results and Discussion

5.3.1 Factors affecting the efficiency of cyanobacterial transformation

Transformation of *Synechococcus* R2 and *Synechococcus* R2-SPc was performed as described by Kuhlemeier *et al.* (1981) - see Section 2.20. Concentrations of antibiotic used for selection were described in Table 2.10. Various modifications of this transformation procedure were performed, including i) the use of stationary phase grown cells, ii) iron-stressed cells as recipients for DNA, and iii) uptake of DNA in the dark.

Various workers have described the existence of a phase during the growth cycle in which the cells are especially competent (see Porter, 1986). However, we found no significant effect on transformation efficiency between exponential phase or stationary phase cells, used as recipients of DNA.

Growth of cells in iron-deficient medium before transformation did not enhance transformation, and may have slightly reduced the efficiency (see Table 5.3). This is in contrast to the observations of Golden and Sherman (1984), who observed a 4-fold increase in efficiency using iron-stressed cells. Their results however, used the wild-type *Synechococcus* R2 strain, whereas work described in Table 5.3 was performed using the small plasmid cured strain. We have obtained consistently higher transformation frequencies using *Synechococcus* R2-SPc, than the wild type strain (see section 7.3.1), which is in contrast to other workers (see Chauvat *et al.*, 1983).

Table 5.3 Affects of variation in procedure on transformation efficiency in *Synechococcus* R2-SPc

Conditions	Plasmid/Selection		Transformants $\mu g\ DNA^{-1}$
a) Exponential phase culture; light DNA uptake; normal medium	pUC303	Cm^r	5.5×10^5
	pLAH4	Cm^r	2.3×10^5
	pLAH4Sal	Cm^r	2.6×10^3
Exponential phase culture; dark DNA uptake; normal medium	pUC303	Cm^r	$10^5 \times 10^6$
	pLAH4Sal	Cm^r	5.2×10^2
b) Stationary phase culture; light DNA uptake; normal medium	pUC303	Cm^r	10^5
	pUC105	Cm^r	10^5
	pUC105	Ap^r	10^4-10^5
Stationary phase culture; light DNA uptake; iron-deficient medium	pUC303	Cm^r	10^4-10^5
	pUC105	Cm^r	10^4
	pUC105	Ap^r	5×10^3-10^4

The effect of a dark treatment after addition of donor DNA and prior to plating on non-selective media, has been shown to enhance (Golden and Sherman, 1984) or cause no difference (Chauvat *et al.*, 1983) in transformation efficiency. We observed no significant difference in transformation frequency in the presence or absence of this dark uptake step for plasmids capable of replicating in *Synechococcus* R2. However, for integrative vectors this frequency decreased approx. 5-fold. Indeed, Williams and Szalay (1983) showed that transformation of *Synechococcus* R2 with non-replicating plasmids that must recombine with the genome, indicated that incubation in the dark during DNA uptake resulted in a greater than 10-fold reduction in transformation efficiency.

It appears then, that even when all factors are taken into account, there still remains considerable variation in transformation efficiency. This can only be partly explained by the different procedures used in different laboratories.

5.3.2 Expression of β-galactosidase in *Synechococcus* R2-SPc

Where present, β-galactosidase (LacZ) is known to be located in the cytoplasm of bacteria, and hence the expression of this enzyme in *Synechococcus* R2 could be used to localise enzyme activity in various cellular compartments.

LacZ was introduced into the shuttle vector pUC105, by ligating a 4.2 kb *Eco*RI-*Sal*I fragment from pTEBG3 into *Eco*RI-*Sal*I digested pUC105, containing both *E. coli* and *Synechococcus* R2 replication origins (Fig. 5.2). The resulting vector, pTUC1 - approx. 14 kb in size - was

transformed into *Synechococcus* R2-SPc. Ap^r transformants were obtained at a frequency of 10^2-10^3 μg DNA^{-1}, and these showed LacZ activity, as detected by spraying plates with MUG (see Fig. 5.2).

Since *E. coli* vectors are not capable of endogenous replication in cyanobacteria (section 5.1.5), we attempted to create a universal recipient strain of *Synechococcus* R2. This used a fragment of non-essential *Synechococcus* R2 chromosomal DNA, to put the entire pBR325 sequence into the chromosome, and then asked the resulting strain to accept, by homologous recombination, a second pBR325 derivative. Plasmid plAH325 was constructed by inserting a 3.7 kb *Hind*III *Synechococcus* R2 chromosomal DNA fragment from plAH4 (a gift of S. Shestakov, see Fig. 5.3) into *Hind*III digested pBR325, and recombinants selected for Ap^r Tc^s (cloning of fragments into the *Hind*III site of pBR325 abolishes tetracycline resistance). Approximately 25% of recombinants were Tc^s. Plasmid plAH325 transformed *Synechococcus* R2-SPc to ampicillin and chloramphenicol resistance at a frequency of 10^3-10^4 transformants μg DNA^{-1}. plAH325 was further modified to ampicillin sensitivity, by inserting *E. coli* strain DH1 chromosomal DNA into the *Pst*I site of plAH325. This then allowed secondary transformation with pBR322 or pBR325 derivatives selecting for Ap^r from the incoming plasmid.

Synechococcus R2-SPc was transformed with this Ap^s plAH325 and the resulting Cm^r transformants used as recipients for transformation by various pBR322-based plasmids, e.g. pTEBG3 (containing *lacZ*), pHCP2 (containing *lamB*) and pPEL (containing the pectate lyase structural gene). Ap^r transformants resulting from integration of the pBR322

vector into the *Synechococcus* R2 chromosome via homologous recombination with Ap^R plAH325, occurred at a frequancy of 5×10^1 - 3×10^2 transformants $\mu g\ DNA^{-1}$. Control experiments, plating Ap^R plAH325 onto solid medium and ampicillin, or transformation of *Synechococcus* R2-SPc lacking Ap^R plAH325, directly, with the pBR322-based vectors, were negative. However, of the three pBR322-based vectors used in the secondary transformation step, only *Synechococcus* R2-SPc containing pTEBG3 :: plAH325 derivatives retained ampicillin resistance when restreaked onto solid medium or in liquid medium, containing antibiotic. Why this is so is not known. *Synechococcus* R2-SPc containing pTEBG3::plAH325 derivatives expressed LacZ activity, as detected by spraying plates with MUG, whereas *Synechococcus* R2-SPc containing plAH325 Ap^R showed no LacZ ctivity. Southern blotting of total DNA from pTEBG3::plAH325 and pTUC1-containing *Synechococcus* R2-SPc and using a 3.95 kb *Bam*H1-*Eco*RI *Lac*Z fragment from pDAH221 (see section 7.3.1) as probe, confirmed the presence of *Lac*Z in these transformants (Fig. 5.4). No homologous hybridising sequences were found in total DNA isolated from plAH325 Ap^R *Synechococcus* R2-SPc.

A similar Ap^R pBR325 derivative, capable of integrating into the cyanobacterial chromosome, was constructed by inserting random *Pst*I fragments of *Synechococcus* R2 chromosomal DNA into *Pst*I cut pBR325. This partial gene library conferred Cm^r to *Synechococcus* R2 transformants, and localised pBR325 sequences to various positions in the chromosome, via homologous recombination. Such a recipient strain was used to insert Tn5 into *Synechococcus* R2, via plasmid pSUP2021 which contains both Tn5 and pBR325 sequences. Expression of the neomycin phosphotransferase gene was observed as detected by resistance to

Figure 5.2

a) Construction of pTUC1, a shuttle vector capable of replication, and *lacZ* expression in *E. coli* and *Synechococcus* R2

b) pTUC1 LacZ activity in *Synechococcus* R2, as detected using MUG

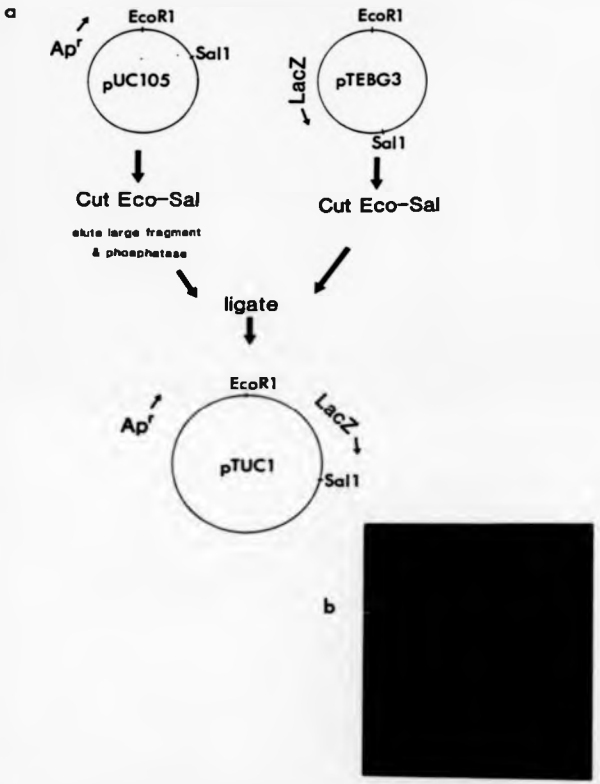


Figure 5.3 Integrative vectors for use in *Synechococcus* R2

a) Restriction maps of the integrative vectors pIAH4, pIAH4Sal and pIAH325, and the *E. coli* lamB-containing plasmid pHCP2

b) 0.7% agarose gel. Lane 1 - *Pst*I cut *Synechococcus* R2 chromosomal DNA::pAR325 (single colony); lane 2 - uncut *Synechococcus* R2 chromosomal DNA::pAR325 (single colony); lane 3 - *Pst*I cut *Synechococcus* R2 chromosomal DNA::pAR325 (total colonies); lane 4 - uncut *Synechococcus* R2 chromosomal DNA::pAR325 (total colonies); lane 5 - *Hind*III cut pIAH325; lane 6 - uncut pIAH325

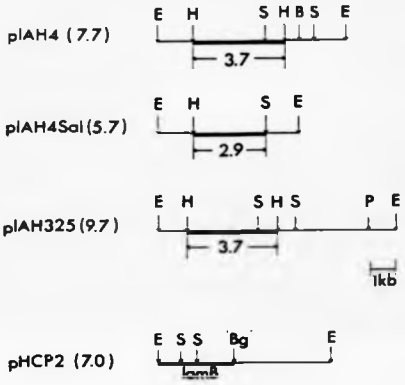
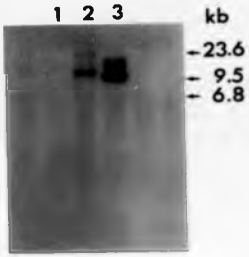


Figure 5.4 Detection of *lacZ* sequences in *Synechococcus* R2-SPc by Southern blotting analysis

Filters were probed with a 3.95 kb *Bam*HI - *Eco*RI *lacZ* fragment from pDAH221 (section 7.3.1) and hybridised at 65°C in 5 x SSPE, 5 x Denhardts, 0.1% SDS overnight. Filters were washed overnight at 65°C in 0.1 x SSPE, 0.1% SDS. Lane 1 - *Pst*I cut total DNA from *Synechococcus* R2-SPc Ap^a pLAH325; lane 2 - *Pst*I cut total DNA from *Synechococcus* R2 Ap^a pLAH325::pTEBG3 derivatives; lane 3 - *Pst*I cut total DNA from *Synechococcus* R2-SPc containing pTUC1.



kanamycin (see section 6.3.2).

Fluorimetric MUG assays of β-galactosidase activity, showed that cytoplasmic fractions from a stationary phase culture of *Synechococcus* R2-SPc containing pTUC1 had an activity of 4.7 picomoles MUG hydrolysed ml^{-1} min^{-1}, compared to 1.5 picomoles MUG hydrolysed ml^{-1} min^{-1} from cytoplasmic fractions of *Synechococcus* R2-SPc containing pTEBG3::plAH325. Activities were normalised for culture density. No activity was detectable in periplasmic or supernatant fractions of these organisms, which is consistent with β-galactosidase being located in the cytoplasm. No data was obtained on the variation in expression of β-galactosidase through the growth phase, and since chromosome copy number and plasmid copy number per cell may change from exponential phase to stationary phase, we cannot directly compare the β-galactosidase activity from a plasmid or chromosomal location. In addition, this type of analysis is complicated by the fact that it is known that the location of a gene within the chromosome can affect its expression - chromosomal position affects expression of the *lac* genes in *E. coli* (see Schmid and Roth, 1987).

5.3.3 Alkaline phosphatase: use as a periplasmic marker, and attempts at gene cloning

Alkaline phosphatase (PhoA) is a known periplasmic enzyme in *E. coli*, and being easily assayed it was a useful marker for the assessment of cell lysis by assaying enzyme activity in the various cellular compartments. Assay of endogenous *Synechococcus* R2 and *Nostoc* sp. MAC PhoA activity was performed as described in section 5.2.2, and this showed that PhoA activity was primarily located in the periplasm (see

Table 5.4). *Nostoc* sp. MAC grown in chelator-deficient medium showed reproducibly higher levels of PhoA in the supernatant than grown in normal medium ± EDDA, but whether this reflected an export event, or simply indicated decreased cell viability, and hence greater cell lysis, was not known.

Alkaline phosphatase from *E. coli* was introduced into *Synechococcus* R2-SPc (see Section 6.3.1) using a cyanobacterial shuttle vector containing Tn*phoA*. If this transposon was to be effectively used to study protein export, we required a Pho^+ strain of *Synechococcus* R2. To achieve this, we attempted to clone the PhoA structural gene, so that by interposon mutagenesis (Fig. 5.5) a Pho^+ strain could be constructed. A 1.3 kb *Xho*I-*Eco*RI internal *pho*A fragment from Tn*phoA* was used to probe *Hind*III and *Eco*RI cut *Nostoc* sp. MAC chromosomal DNA and *Bam* HI, *Eco*I cut *Synechococcus* R2 chromosomal DNA. After hybridising at 65°C in 6 x SSPE, filters were washed consecutively in i) 2 x SSPE at room temperature and 6 x SSPE at 65°C for 1 hr, ii) 2 x SSPE at 65°C overnight, and iii) 1 x SSPE at 65°C overnight. Autoradiographs were developed at each stage. Although some hybridisation was noted at lower stringencies the signal was thought not sufficiently above background to be of use in screening by colony or plaque hybridisation.

A complete gene library of *Synechococcus* R2 was constructed by inserting partially *Sau* 3A digested *Synechococcus* R2 chromosomal DNA (fragment size 4 kb) into *Bam*HI digested pBR322. This library was transformed into *E. coli* strain CC118 ($PhoA^-$) and transformants plated onto nutrient agar + ampicillin + XP (the chromogenic substrate for PhoA). All bacterial colonies (10^3-10^4) were white, indicating no PhoA activity,

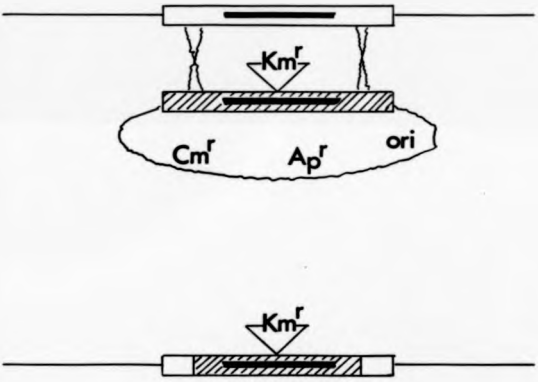
Table 5.4 Localisation of PhoA in *Synechococcus* R2 and *Nostoc* sp MAC

Strain/Growth conditions	PhoA activity ΔOD_{420} min^{-1} ml^{-1} mg $protein^{-1}$		
	lysate	periplasm	supernatant
Nostoc sp MAC			
normal medium	1.2 (24%)	3.4 (71%)	0.2 (4%)
normal medium +EDDA	1.6 (14%)	9.3 (79.5%)	0.76 (6.5%)
chelator-deficient medium	2.6 (25%)	6.8 (65%)	1.1 (10%)
Synechococcus R2			
normal medium	1.1 (8%)	13 (91%)	0.02 (0.15%)
E. coli strain DH1	2.8 (14%)	15.5 (77%)	1.6 (8%)

The figure in brackets is the percentage of total activity in i) lysate, ii) periplasm, iii) supernatant. (see also Table 7.3)

Figure 5.5 Mechanism by which chromosomal genes can be inactivated by transformation

The shaded bar represents a *Synechococcus* gene cloned into pBR322, further modified by insertion of Tn5 carrying a gene for kanamycin resistance (Km^r). The open bar represents the corresponding wild type gene in the chromosome of *Synechococcus*. Transformation with plasmid DNA, selecting for kanamycin resistance, results in reciprocal recombination or gene conversion which replaces the wild type gene with the inactivated allele. Transcription of the gene is abortive.



and suggesting that either a complete *phoA* gene was not present in the library, or that *Synehcococcus* R2 *phoA* was not expressed in *E. coli*. The use of cosmid libraries of cyanobacterial chromosomal DNA, with larger DNA inserts, may be more useful in this respect, assuming the cyanobacterial *phoA* is expressed in *E. coli*.

The introduction of *lamB* into *Synechococcus* R2 is of interest due to the ability of the gene product, an outer membrane protein receptor, to allow infection by phage lambda. This would be of importance in mutagenesis studies (see Chapter 6). We have constructed a partial gene library of *Synechococcus* R2 chromosomal DNA in the *Eco*RI site of pHCP2 and this now awaits transformation into *Synechococcus* and analysis of expression.

5.3.4 Introduction of the gene encoding pectate lyase, into *Synechococcus* R2-SPc

Pectate lyase is one of the extracellular degradative enzymes secreted by *Erwinia* sp. which attack the middle lamellae and the primary cell wall of plant tissues, and cause soft rot in a number of economically important crop plants (see Hinton and Salmond, 1987). Since the enzyme was secreted into the culture medium in *Erwinia* sp. and easily assayed, it was a good candidate for introduction into *Synechococcus* R2, to assess the expression and export of a foreign protein by a cyanobacterium.

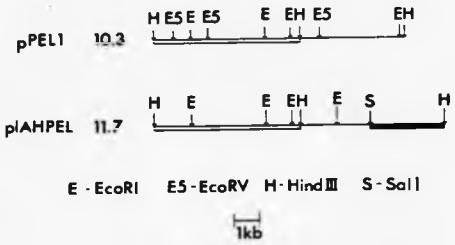
Plasmid pPEL1 (a gift of J. Hinton, this laboratory) is a pBR322 based vector containing a 6 kb *Hind*III fragment which carries the pectate lyase structural gene. Since the pectate lyase gene fragment could not

easily be subcloned into a cyanobacterial shuttle vector, an integrative vector system was used to introduce this fragment into the cyanobacterial chromosome. plAH4 (a gift of S. Shestakov) contains a 3.7 kb *Synechococcus* R2 chromosomal DNA fragment in the *E. coli* vector pACYC184. This vector was modified to remove one of the *Hind*III sites, by deletion of a 2 kb *Sal*I fragment. The resulting plasmid, plAH4Sal, contained a single *Hind*III site, and a 2.9 kb *Synechococcus* R2 chromosomal DNA fragment. The removal of one of the Hind III sites of plAH4 allowed direct insertion of the 6 kb *Hind*III fragment encoding pectate lyase, into the single *Hind*III site of plAH4Sal. The isolated 6 kb *Hind*III fragment was ligated with phosphatased *Hind*III cut plAH4Sal, and transformants in *E. coli* strain DH1 selected for Cm^r. 24 Cm^r transformants were obtained, and all showed pectate lyase activity in *E. coli* as detected by plate assay (see Fig. 5.7). Restriction enzyme analysis of plasmid DNA from 10 of these transformants gave a similar restriction pattern (Fig. 5.6), suggesting the orientation of the insert was the same. This plasmid, designated plAHPEL, approx. 12 kb in size, was transformed into *Synechococcus* R2-SPc. Cm^r transformants occurred at a frequency of 1.5×10^3 μg DNA^{-1} (for comparison with plAH4 and plAH4Sal see Table 5.3). Several *Synechococcus* R2-SPc transformants were grown in liquid containing 5 μg ml^{-1} chloramphenicol, and cytoplasmic and supernatant fractions assayed for pectate lyase activity. No activity was detectable as assessed by spectrophotometric means (section 5.2.3). However, total DNA isolated from Cm^r *Synechococcus* R2-SPc transformants showed homologous hybridising sequences to both plAH4, and the pPEL1 6 kb *Hind*III fragment, when used as probes in Southern hybridisation experiments (see Fig. 5.7). This suggested that the pectate lyase gene was i) present in

Figure 5.6
a) Restriction maps of plAHPEL and pPEL1

b) 0.7% agarose gel

Lane 1 - *Eco*RI-*Hind*III cut plAHPEL; lane 2 - *Hind*III cut plAHPEL; lane 3 - *Eco*RI cut plAHPEL; lane 4 - *Hind*III cut plAH6Sal; lane 5 - *Hind*III cut pBR325; lane 6 - *Hind*III cut plAH325.



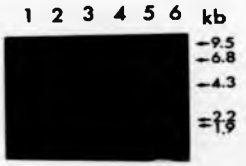


Figure 5.7

a) Pectate lyase activity by *E. coli* strain DH1 transformants containing pLAHPEL. Arrows indicate negative controls, lacking pLAHPEL.

b) Detection of *pel* and pACYC184 DNA sequences in *Synechococcus* R2 by Southern blotting analysis.

Filters were probed with i) pLAH4 - lanes 1-4, and ii) a 6 kb Hind III fragment from pPEL containing the pectate lyase structural gene - lanes 5-8. Hybridisation and washing conditions were as described in Figure 5.4. Lanes 1 and 5 - *Hind*III cut *Synechococcus* R2 chromosomal DNA; lanes 2-4 and 6-8 - *Hind*III cut *Synechococcus* R2 chromosomal DNA::pLAHPEL from 3 individual transformants.

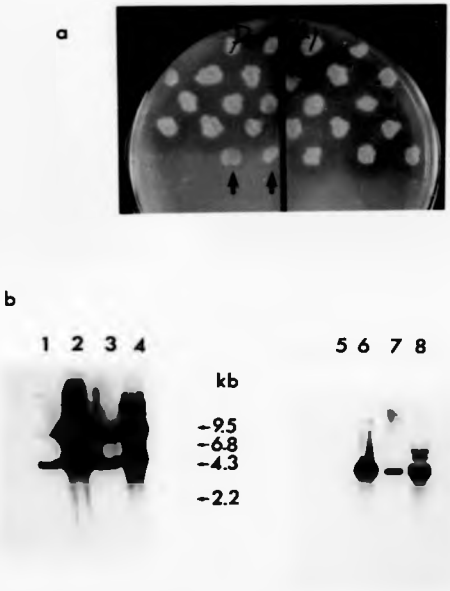


Table 5.5 Spectrophotometric analysis of pectate lyase activity in *E. coli* strain DH1 and *Ecc* strain HC1031

Strain	$\Delta A_{235}\ min^{-1}\ ml^{-1}$ mg protein^{-1}
Ecc HC1031 supernatant	39,470
E. coli strain DH1 (plAHPEL) supernatant	2,060
E. coli strain DH1 (plAHPEL) lysate	2,660
E. coli strain DH1 (plAHPEL) lysate +	
i) *Synechococcus* R2 lysate 1 µl	1,180
ii) *Synechococcus* R2 lysate 2 µl	1,062
iii) *Synechococcus* R2 lysate 5 µl	963
iv) *Synechococcus* R2 lysate 10 µl	292
v) *Synechococcus* R2 lysate 15 µl	108
vi) *Synechococcus* R2 lysate 20 µl	zero
vii) *Synechococcus* R2 supt. 20 µl	2,550
viii) Allen's medium 20 µl	2,100
ix) *Synechococcus* R2 lysate boiled 20 µl	40

the chromosome, but not expressed, ii) expressed at very low levels, but the active enzyme not detectable by spectrophotometry, or iii) expressed at normal levels but the enzyme rapidly degraded, or an inactive enzyme produced. To assess whether the pectate lyase gene was being transcribed, Northern blotting analysis was performed on total RNA isolated from transformants, and using the 6 kb pectate lyase encoded DNA fragment as probe. No hybridisation was detectable. This suggested that the gene was either not transcribed, or that the levels of mRNA were too low for an adequate signal to be detected.

E. coli strain DH1 containing plAHPEL had only 10-20% pectate lyase activity as compared to *Erwinia carotovora* subsp. *carotovora* (Ecc) strain HC1031, and of this activity a variable amount (20-50%) was extracellular. Whether pectate lyase was actively secreted by *E. coli* or the enzyme appeared in the supernatant through leakage from the periplasm or by cell lysis requires more critical examination. Reverchon *et al.* (1986) showed that five isoenzymes of pectate lyase from *Erwinia chrysanthemi* were mainly periplasmically located in *E. coli*. Although pectate lyase activity varies widely in various *E. coli* strains compared to the wild type *Erwinia* sp., recent data (Hinton and Salmond, 1987) suggests that the growth temperature significantly affects enzyme synthesis, with little or no enzyme synthesis by *Ecc* at 37°C compared to 30°C, the normal growth temperature. Since *E. coli* strain DH1 was routinely grown at 37°C this is an important consideration.

The pectate lyase activity of *E. coli* strain DH1 containing plAHPEL, or of *Ecc* strain HC1031 was reduced or abolished by the addition of

Synechococcus R2 cell lysate (see Table 5.5), as assessed by spectrophotometric analysis. The pH of the reaction mixture was not affected by addition of the cyanobacterial lysate, and addition of calcium ions, the only known requirement for enzyme activity, in vast excess, did not reverse the inhibition process. Boiled cyanobacterial lysate caused a similar inhibition, suggesting that either a protein was not involved, or that the 'inhibitor' was heat stable. These results do not exclude the possibility of some non-specific inhibition of enzyme activity, although *Synechococcus* R2 supernatant or Allen's medium had little or no affect on enzyme activity (Table 5.5). However, similar experiments using pectate lyase assay plates, showed normal enzyme activities. It could be that whatever was causing inhibition in the spectrophotometric assay was either diffusing out through the plate and allowing the enzyme to act, or that the solid media was providing some factor allowing enzyme activity.

5.3.5 Conjugal gene transfer into *Nostoc* sp. MAC

The construction of a biparental mating system for transfer of genes from *E. coli* to *Anabaena* sp. ATCC 27893 (MacFarlane *et al.*, 1987) allowed all conjugation functions to be present in one *E. coli* strain, and thus differs from the triparental mating system developed by Wolk *et al.* (1984). We have constructed a similar strain containing pVWIC (Ap^R, Cm^R), capable of replicating in various filamentous cyanobacteria and pGJ28 a helper plasmid providing necessary transfer functions, transformed into *E. coli* S17.1. This strain was conjugated to *Nostoc* sp. MAC PCC8009 (see section 5.2.4), and transconjugants selected for Cm^R (50 $\mu g\ ml^{-1}$). After growth of transconjugants in liquid medium, the *E. coli* donor strain no longer grew, and this was adequate to produce a

'clean' culture. Transconjugants were also selected for Ap^R, but because of a high endogenous resistance to both ampicillin and chloramphenicol (15 $\mu g\ ml^{-1}$), and a considerably reduced growth rate, it was not feasible to determine the conjugation frequency.

Mating of *E. coli* S17.1 containing pRL6 and pDS4101 to *Nostoc* sp. MAC also produced Cm^R transconjugants. Interestingly, plasmid DNA isolated from transconjugants (M. R. K. Alley, this laboratory) showed that pRL6 had lost one of its *Bgl*II sites. This rearrangement might be expected since, as mentioned in section 5.1.2, *Nostoc* sp. MAC produces a restriction enzyme Nsp. MACI which is an isoschizomer of *Bgl*II. Hence, plasmid DNA should be expected to lose at least one of these *Bgl*II sites.

5.4 Conclusion

By using enzymes known to be located in cytoplasmic and periplasmic compartments of the bacterial cell, we have shown that no obvious contamination of supernatant fractions was detectable. This suggests a true export of proteins in these cyanobacteria (see Chapter 3). The expression of β-galactosidase in *Synechococcus* R2, as well as being a good cytoplasmic marker, in future allows a detailed analysis of cyanobacterial gene expression, through gene fusion technology (and see Chapter 7).

The gene encoding the extracellular enzyme, pectate lyase was introduced into the *Synechococcus* R2 chromosome by linking the foreign DNA to the ends of a *Synechococcus* R2 DNA fragment, which allowed integration via

homologous recombination in type II and type III configurations (see Fig. 5.1). Upon maintained selection, stable Cm^r transformants were produced, but no enzyme activity was detectable. This may have been a result of some form of 'inhibition'. The potential use of cyanobacteria as 'exporters' of foreign proteins thus still remains to be determined.

Preliminary experiments have shown that *Nostoc* sp. MAC is capable of being conjugated using vectors developed by Wolk *et al.* (1984). This opens up the possibilities for genetic manipulation of this organism.

Chapter 6

Use of Transposon and Chemical Mutagenesis to study Cyanobacterial Protein Export

6.1 Introduction

Stable mutants of cyanobacteria have been obtained by various mutation induction techniques, including the use of chemical mutagens, ultraviolet light and x-rays. Mutants resistant to antibiotics and other compounds, temperature-sensitive mutants, auxotrophic mutants, mutants defective in photosynthetic pigments or activity and mutants altered in cellular differentiation have all been obtained (for review see Herdman, 1982). It was thought that the difficulty encountered in isolating auxotrophic mutants of cyanobacteria suggested these organisms were particularly resistant to mutagens, however, it became evident that the effectiveness of various mutagens was strain-dependent. Where studied, mutagens appear to affect cyanobacterial DNA in exactly the same way as in other organisms - ultraviolet irradiation induces the formation of thymine dimers whilst ethyl methane sulphonate (EMS), and N-methyl-N'-nitro-N-nitrosoguanidine (NTG) act specifically at the DNA replication fork. Although NTG has been the most widely used mutagen in cyanobacteria, there is a risk of inducing multiple mutations at closely linked sites, and hence care must be taken in interpreting the nature of mutants isolated following exposure to this chemical. Whilst we have used NTG as a mutagen in this study, we were interested in developing a more transposon-based mutagenesis protocol, and to this end have attempted to further develop a system of transposon mutagenesis for use in unicellular cyanobacteria. The recent development of a transposon probe, Tn*phoA*, which can be used to identify mutants of *E. coli* carrying mutations in genes encoding exported proteins, has stimulated much interest in this area (see section 6.1.). The advantages of transposon mutagenesis over conventional mutagenesis techniques are discussed below.

6.1.2 What are transposons?

Transposons are mobile genetic elements which can integrate or translocate, from one replicon to another. The elements differ in the degree of specificity which they display; some appear to insert randomly whereas others have distinct preferences for particular sites. In all cases, however, insertion is independent of homologous recombination. Transposons carry genes necessary for maintenance and transposition, and often encode resistance to antibiotics. A number of transposons have now been described (Kleckner, 1981) - see Table 6.1. These have been classified into two basic arrangements. Class I transposons contain a drug resistant determinant flanked by copies of an IS element either as direct, or more commonly, as inverted repeats (IR). The IS elements provide the transposition functions and act in concert to transpose the intervening drug resistance gene(s). In the second arrangement a sequence is flanked by short inverted repeats, typically 30 to 40 bp. Between the IRs are genes encoding transposition functions as well as those encoding drug resistance. Well over 50 transposons of Class I and II have now been identified (Kleckner, 1981).

Thus there are a wide range of transposons encoding antibiotic resistance, or resistance to heavy metal ions, which might be used to mutagenize cyanobacteria.

The properties of transposons we were interested in, which are particularly important for their use in bacterial genetics, are i) that they can insert at a large number of sites on the bacterial chromosome, ii) that such insertions can generate stable mutations by inactivation of a gene (interrupted genes suffer complete loss of function),

Table 6.1 Class I and II Transposons

Transposons	Markers	Ends
Class I		
Tn5	Km	IS50 IR
Tn9	Cm	IS1 DR
Tn10	Tc	IS10 IR
Tn903	Km	IS903 IR
Tn1681	Stable enterotoxin	IS1 IR
Class II		
Tn1	Ap	38bp IR
Tn901	Ap	38bp IR
Tn7	Tp, Sm	19bp IR
Tn501	Hg	38bp IR
Tn551	Ery	35bp IR
Tn1721	Tc	38bp IR
Tn1000 (γ δ)	none	37bp IR
Tn4	Sm, Su, Ap	<140bp IR
Tn21	Sm, Su, Hg	38bp IR
Tn2603	Sm, Su, Hg, Oxa	<140bp IR

IR, inverted repeat; DR, direct repeat; bp, base pair

iii) that such insertion mutations provide a simultaneous antibiotic selection, this allows the marking and cloning of non-selectable genes, iv) that mutants are produced with the very high probability of only a single insertion event having occurred in that gene, and hence that there is no problem of the multiple mutational events which are associated with using NTG.

6.1.3 Transposons and cyanobacteria

van den Hondel *et al.* (1980) reported the introduction of the ampicillin resistance transposon Tn901 into the small resident plasmid of *Synechococcus* R2. Tn901 is a 4.5 kb transposable element that encodes resistance to ampicillin. Transformation of *Synechococcus* R2 with pRI46 (pRI4665 :: Tn901) produced colonies containing plasmids consisting of the complete sequence of Tn901 inserted at different places in the small plasmid pUH24. Such recombinant plasmids (pUH24 :: Tn901) have become the basis for some of the host-vector systems now available for this organism.

Tandeau de Marsac *et al* (1982) furthered the use of transposon technology in cyanobacteria by using transposon Tn901 to clone a gene involved in methionine biosynthesis in *Synechococcus* R2. After isolating a *met* :: Tn901 mutant of *Synechococcus* R2 a recombinant plasmid carrying the inactivated *met* :: Tn901 gene was selected after transformation into *E. coli*. The cloned met :: Tn901 DNA fragment was used as a probe to select the corresponding *Synechococcus* R2 wild type *met* gene from a gene library prepared in *E. coli*.

Since the transposition frequency of Tn901 in cyanobacteria is low however, workers have attempted to find more suitable/efficient transposable elements with which to mutagenise the cyanobacterial genome.

Bullerjahn (1985) constructed a plasmid containing the colE1 *ori*, RP4 *trs* functions, and Tn501, a 7.9 kb transposon that confers resistance to mercuric ions. It was demonstrated that low concentrations of Hg^{2+} promoted transposition into *Aphanocapsa* PCC6714; 10 μM Hg^{2+} present during conjugation increased the frequency of Hg^{2+}-resistant exconjugants 5-fold. He also showed by Southern blots that Tn501 had inserted into the chromosome. However, resistant colonies attained a 'sectored' appearance suggesting the instability of such insertions (G. Bullerjahn - personal communication).

We have attempted to mutagenise *Synechococcus* R2 using the 5.7 kb transposon Tn5 (see section 6.3.2) which encodes kanamycin and neomycin resistance (and streptomycin resistance in certain bacterial species). The properties of Tn5 have been exhaustively studied (for review see de Bruijn and Lupski, 1984), and it has been used successfully in the genetic analysis of a number of bacterial species. This is discussed below.

6.1.4 Transposon Tn5

Tn5-induced insertion mutations have been generated in a wide variety of chromosomal genes and operons of *E. coli* and *Salmonella typhimurium* such as *his* and *lac*. In addition, Tn5 mutagenesis has been used to generate mutations and then to facilitate cloning genes of several bacterial

species such as *Caulobacter crescentus*, *Myxococcus xanthus* and *Erwinia carotovora* subsp. *carotovora* (see Hinton, 1986).

Tn5 has a number of advantages over other transposons in that i) it has a high transposition frequency - 10^{-2} to 10^{-3} per cell in *E. coli*; ii) it has a relatively low insertional specificity, i.e. it appears to insert randomly, or at least randomly enough to make it useful for insertion mutagenesis of cloned DNA sequences (Berg and Berg, 1983); iii) it causes mutations which exert a strong polar effect on genes located distally (downstream) to the insertion site, within the same operon, due to transcriptional termination signals carried by the Tn-element (de Bruijn and Lupski, 1984); iv) it has been extensively restriction mapped, and v) produces relatively stable insertions. In addition, kanamycin resistance provides a better means of selection in cyanobacteria than the ampicillin-resistance encoded by Tn901.

6.1.5 Vectors used for transposon Tn5 mutagenesis

In order to use a transposon as a mutagenic agent, a replicon is needed that both carries the transposon and can be efficiently introduced into the organism to be mutagenised. Once in the recipient strain, however, a means of selecting against stabilisation of the transposon carrier replicon is required. The use of such so-called "suicide-vectors" - unstable transposon delivery vehicles which fail to replicate in the recipient - allows selection for transposon mediated antibiotic resistance and ensures survivors contain transposon inserts.

pJB4JI (Beringer *et al.*, 1978) is one such suicide plasmid - a promiscuous P1-type R-factor (RP4) carrying a copy of bacteriophage Mu

and of Tn5. The plasmid is extremely unstable in such bacterial genera as *Rhizobium*, *Agrobacterium*, *Caulobacter* and *Erwinia*, due to Mu-mediated illegitimate recombination events. Selection for Km^R or Nm^R after mating the plasmid across from *E. coli* results in the transposition of the Tn5 from the suicide plasmid into the genome of the recipient cell, followed by loss of the plasmid vector DNA sequences. However, some authors have reported the insertion of phage Mu DNA sequences in addition to Tn5 sequences at the site of insertion (Meade *et al.*, 1982) and hence presumptive Tn5 induced insertion mutations should be checked for the presence of both Tn5 and Mu DNA sequences by Southern blotting. It has also been noted that the applicability of pJB4JI varies dramatically from strain to strain (Zink *et al.*, 1984).

An alternative vector, pSUP2021 (Simon *et al.*, 1983), has been constructed and consists of the pBR325 replicon into which the mobilization (*mob*) site of the promiscuous P-type plasmid RP4 has been inserted and carrying a copy of Tn5. (The *mob* site includes the origin of transfer (*oriT*) and acts as a recognition site for certain trans-acting RP4 transfer functions). Such a plasmid has been mobilized at very high frequencies to bacterial species such as *Rhizobium* or *Agrobacterium*, when provided in *trans* with transfer functions. This has relied on the use of broad host range mobilizing strains in which the RP4 plasmid is integrated into the *E. coli* chromosome, e.g. S17.1, used in this study. It has been suggested that the use of pSUP2021 may circumvent some of the problems associated with pJB4JI (de Bruijn and Lupski, 1984).

Tn5 mutagenesis using bacteriophage vectors has also been used, in *E. coli*, *S. typhimurium* and *Myxococcus xanthus*. In *S. typhimurium* a P22 :: Tn5 derivative was used, whilst in *Myxococcus* bacteriophage Fl :: Tn5 was employed, both bacteriophage vectors allowing adsorption and injection of phage DNA, but incapable of replicating in the respective bacteria.

In bacterial species sensitive to infection by phage λ, a λ :: Tn5 derivative has been proven to be most effective. We have already considered our attempts to introduce the *E. coli malB* region, encoding the λ receptor protein (*lamB*) into cyanobacteria (see section 5.3.3), and this has been successful in several *Erwinia* strains (Salmond *et al.*, 1986) and in *S. typhimurium*. The *LamB* protein alone allows λ phage adsorption and DNA injection, but not replication. λ suicide vectors for transposon mutagenesis have significant advantages, e.g. the availability of many transposon-carrying λ-derivatives (Berg and Berg, 1983; Manoil and Beckwith, 1985), including λ :: Tn*phoA* developed as a probe for protein export signals. Furthermore, phage infection is rapid, and allows random transposon induced mutation without problems of sibling formation (Hinton, 1986).

It can be seen then, that a variety of Tn5 derivatives have been used, and no one transposon delivery system has been universally applicable.

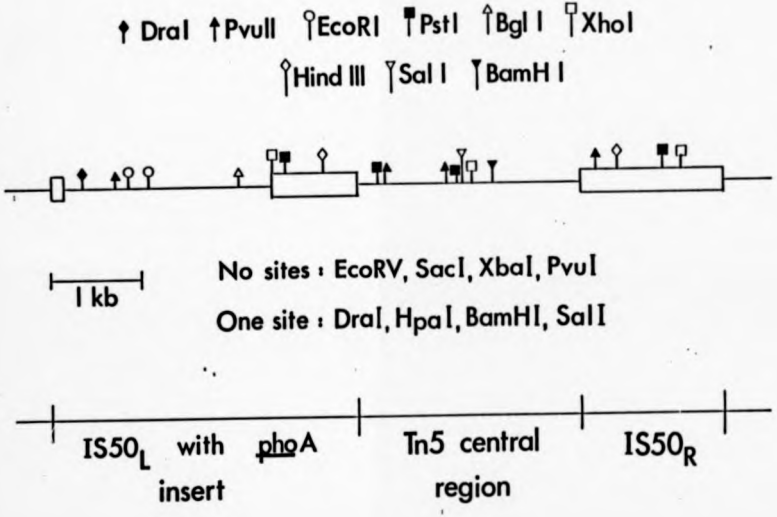
Since we were particularly interested in developing a system for detecting mutants defective in protein export, the availability of Tn*phoA*, a transposon probe for protein export signals (Manoil and Beckwith, 1985), allowed us to investigate its use in cyanobacteria.

6.1.6 Transposon Tn*phoA*

Protein fusions have played a central role in molecular genetic studies of the mechanism of protein export in bacteria (Michaelis and Beckwith, 1982, section 7.1.3), and have relied on the fusion of a specific assayable enzyme to the amino-terminal sequences of an exported protein. Hoffman and Wright (1985) constructed a series of vectors which could be manipulated *in vitro* to fuse the gene for the *E. coli* periplasmic protein alkaline phosphatase (EC 3.1.3.1 PhoA) to different cloned genes. They altered the *phoA* gene, removing its promoter and signal-sequence encoding region but retaining enough of the mature protein that highly active alkaline phosphatase could be generated by the fusions. A variety of hybrids containing protein export signals attached to PhoA were secreted and showed enzyme activity. Since PhoA activity is absolutely dependant on its export to the periplasm, this is a good criterium that active fusions contain sequences which allow the enzyme to be secreted.

Manoil and Beckwith (1985) presented an extension of this approach that allowed the ready isolation of fusions to alkaline phosphatase *in vivo*. They constructed a transposon, Tn*phoA*, which is a Tn5 derivative carrying a truncated version of the *E. coli phoA* gene within IS 50L (see Fig. 6.1). When this transposon inserts into a gene coding for an exported protein, in the correct orientation and reading frame, a hybrid protein is generated, and PhoA activity is seen only if this hybrid is exported to the periplasm. It was interesting that Manoil and Beckwith (1985) showed that PhoA export could be promoted not only by signal sequences of periplasmic proteins, but also by sequences within complex cytoplasmic membrane proteins - a class of trans-membrane proteins

Figure 6.1 Restriction map of TnphoA



generally lacking cleaved signal sequences. Tn*phoA* has since been used to detect genes for exported proteins in *E. coli* (Boquet *et al.*, 1987) and in *Vibrio cholerae* (Taylor *et al.*, 1987). In addition, Hinton and Salmond (1987) have demonstrated the ability of Tn*phoA* to identify extracellular enzyme mutants of *Erwinia carotovora* subsp. *carotovora*.

6.2 Materials and Methods

6.2.1 Preparation of high-titre phage λ lysates

λ :: Tn*phoA* lysates were prepared on the suppressing *E. coli* host LE392 by the method described by Bruijn and Lupski (1984). An overnight *E. coli* culture was grown in LB + 0.2% maltose, 10 mM $MgSO_4$. An aliquot (300 µl) of the culture was infected with 10^5-10^6 pfu of λ :: Tn*phoA* (two fresh plaques resuspended in phage buffer - 7 g Na_2HPO_4 anhydrous, 3 g KH_2PO_4 anhydrous, 5 g NaCl, 2.5 g $MgSO_4.7H_2O$, 1 ml 1% (w/v) gelatin per litre) at room temperature for 10 min. 3 ml soft double Difco agar (0.6% Bacto agar + double Difco medium - see Table 2.4) was added and the mixture overlayed upon a fresh 1% double Difco agar plate. Plates were incubated at 37°C for ca. 8 hr, until confluent lysis was observed. The top agar was removed with a glass spreader, and the plate washed with 3 ml phage buffer. 0.5 ml of chloroform was added, and the mixture vortexed for at least 3 min. Agar was removed by centrifugation in an MSE Chilspin (5000 rpm, 10 min 4°C) and the supernatant decanted. Lysates were stored over a few drops of $CHCl_3$ at 4°C.

6.2.2 Transposon Tn*phoA* insertion into plasmid vectors

Plasmid DNA (pUC105) was transformed into *E. coli* strain CC118 (*phoA*$^-$ λ^R) using the method described in section 2.19. An overnight culture of

CC118 containing plasmid DNA was grown in LB + 0.2% maltose, 10 mM $MgSO_4$ with plasmid selective antibiotic. 0.5 ml of this culture was resuspended into 10 ml fresh medium and incubated at 37°C for 2 hr with shaking. λ :: Tn*phoA* lysate (150 μl) was added to the cell culture, and after brief vortexing, the mixture was incubated at 30°C for 90 min without shaking. (Tn5 transposes with a higher efficiency at 30°C). Cells were harvested by centrifugation in an MSE Chilspin (5000 rpm, 10 min, 4°C) and resuspended in 150 μl LB + 0.2% maltose, 10 mM $MgSO_4$. Infected cells were then plated onto LB agar plates containing 40 μg ml^{-1} XP, and either 300 μg ml^{-1} kanamycin (100 μl cells per plate) or 50 μg ml^{-1} kanamycin (10 μl cells per plate) and incubated at 37°C for 24-48 hr. Uninfected control cultures were plated onto media containing 50 μg ml^{-1} kanamycin. The high concentration of kanamycin (300 μg ml^{-1}) enriches for cells carrying insertions of the transposon into the multicopy plasmid. After 2 days of incubation at 37°C, cells producing blue colonies (with alkaline phosphatase activity) and white colonies (without alkaline phosphatase activity), on agar plates containing 300 μg ml^{-1} kanamycin, were resuspended in nutrient broth + 300 μg ml^{-1} kanamycin. Plasmid DNA was prepared from these cells (section 2.11) after an additional day of growth at 37°C. Plasmids were analysed for loss of gene function (e.g. ampicillin resistance) and by restriction mapping to determine sites of Tn*phoA* insertion.

6.2.3 Conjugation between *E. coli* and cyanobacteria

1 ml of an overnight culture of *E. coli* strain S17.1 containing the appropriate transposon/plasmid was mixed with 1 ml of a late exponential phase culture of *Synechococcus* R2, *Synechococcus* sp. or *Nostoc* sp. MAC respectively, and filtered onto 0.45 μm Millipore filters. The filters

were placed on Allen's medium plates for 24 hr at 30°C in low light (10-15 $\mu E\ m^{-2}\ s^{-1}$) and the cells resuspended in Allen's medium, and plated onto solid medium containing the appropriate selective antibiotic. Presumptive transconjugants appeared after 3-6 days, depending on the strain. Control experiments were identical, except that the *E. coli* strain lacked the appropriate transposon vehicle.

6.2.4 NTG mutagenesis

Synechococcus R2 and *Nostoc* sp. MAC were grown in Allen's medium (100 ml) to early logarithmic phase, and cells resuspended to give a final concentration of 5×10^7 cells ml^{-1}. *Nostoc* sp. MAC filaments were then broken to 3-4 cell filaments average size in a sonicating water bath (ca. 8 min). Cultures were treated with 0.05 $\mu g\ ml^{-1}$ NTG, and incubated at room temperature for 6 hr. Samples (1 ml) were removed every hour and appropriate dilutions spread onto Allen's medium plates to determine the number of survivors. Untreated control cultures were used to obtain viable counts. After 6 hr, cultures were washed free of NTG and transferred to Allen's medium ± added chelator at 34°C for selection of mutants (2-3 days).

6.2.5 Penicillin enrichment

Transposon-mutagenized or NTG-treated cells were grown in selective medium for 2-3 days and enriched for mutants by the addition of penicillin 1 mg ml^{-1}. (Medium containing Tn*phoA* mutagenised cells contained kanamycin 25 $\mu g\ ml^{-1}$, in addition to lacking endogenous chelator). After incubation at 34°C for 1-2 days, the remaining cells were harvested by centrifugation, plated onto Allen's agar and incubated for a further 4-5 days until colonies appeared. Presumptive mutants

were restreaked onto solid medium with and without added chelator.

6.2.6 Selection for chlorate resistance

The lethal dose of chlorate toxicity was determined by adding 0.5-2 ml of a 50% (w/v) stock solution of sodium chlorate, underlayed underneath agar plates containing 20 mM sodium nitrate, 0.1 mM ammonium sulphate to give a final concentration of 0-2% (w/v) sodium chlorate. Agar plates contained appropriately diluted cultures of *Synechococcus* R2 and *Synechococcus* sp. Viable counts of cultures were determined by plating cells onto medium lacking sodium chlorate.

6.3 Results and Discussion

6.3.1 Introduction of Tn*phoA* into pUC105

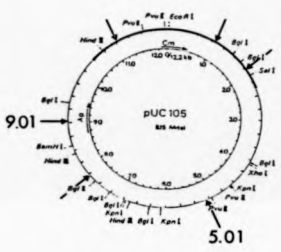
Tn*phoA* was introduced into the shuttle vector pUC105 (capable of endogenous replication in *E. coli* and *Synechococcus* R2) using the method described in section 6.2.2. Thirteen colonies resistant to 300 $\mu g\ ml^{-1}$ kanamycin were obtained, 12 were white and 1 was blue (see Fig. 6.2), the latter indicating alkaline phosphatase activity and suggesting that the transposon had transposed into an area of the plasmid encoding an exported protein. Plasmid DNA was isolated from 5 white and 1 blue colonies, and the sites of Tn*phoA* insertion determined by restriction mapping (see Fig. 6.3). Tn*phoA* inserted randomly around pUC105 which agrees with the findings of Manoil and Beckwith (1985) and suggests that Tn*phoA* retains the low DNA sequence specificity of insertion of Tn5. As expected, restriction enzyme analysis of plasmid DNA isolated from the blue colony, with *phoA* activity, mapped the Tn*phoA* to within the ampicillin resistance gene (*bla*) encoding the periplasmic enzyme

Figure 6.2 Detection of PhoA activity in *E. coli* strain CC118 (blue colonies) due to insertion of TnphoA into a gene encoding an exported protein of pAR322

Figure 6.3 Site of TnphoA insertion into pUC105

a) Restriction map of pUC105 showing sites of insertion of TnphoA (indicated by arrows).

b) 0.7% agarose gel, lane 1 - plasmid 5.01 cut *Xho*I-*Bam*HI; lane 2 - plasmid 5.01 uncut; lane 3 - plasmid 9.01 cut *Xho*I-*Bam*HI. Arrow indicates 4.5 kb *Xho*I-*Bam*HI fragment essential for replication in *Synechococcus* R2; lane 4 - plasmid 9.01 uncut; lane 5 - λ HindIII molecular weight markers (size in kilobases).



β-lactamase. The precise location of Tn*phoA* insertion or the size of the *bla-phoA* hybrid protein was not determined. However, alkaline phosphatase activity implies that a hybrid protein comprising at least the NH_2-terminus from β-lactamase - and hence containing protein export signals, i.e. signal sequences from this enzyme - has fused with the alkaline phosphatase structural gene product. Interestingly, *E. coli* cells containing this plasmid grew poorly in 50 μg ml^{-1} ampicillin, and transformed *Synechococcus* R2 cells were Ap^S. This might suggest that the hybrid protein is unstable, or that a full-length β-lactamase was not formed. In this latter case, long *bla-phoA* hybrid proteins may fractionate to the inner membrane because they lack carboxy-terminal β-lactamase sequences necessary for full solubility, and hence an active β-lactamase capable of conferring Ap^r would not be formed.

The successful insertion of Tn*phoA* into pUC105 allowed, i) a means of introducing this transposon into the cyanobacterial cell, since a suitable suicide vector was not available. (Direct infection of cyanobacterial cells with λ :: Tn*phoA* is not yet possible, though the successful expression of the *lam*B gene (see section 5.3.3) will aid in this process). ii) An assessment of the transposition ability of this transposon in *Synechococcus* R2 and iii) an investigation into the effect of the insertion of Tn5 based inserts on the stability of the cyanobacterial cloning vector.

Two Tn*phoA* containing derivatives of pUC105, insert 5.01 and insert 9.01 (see Fig. 6.3) were transformed into *Synechococcus* R2-SPc (see Table 6.2). Transformants were selected either for the original plasmid resistance marker (Ap^r 1 μg ml^{-1}) or for the resistance marker carried

by the transposon (Km^r 25 $\mu g\ ml^{-1}$). These experiments were carried out with the plasmid-cured strain to avoid the possibility of obtaining homologous recombination with the endogenous plasmid.

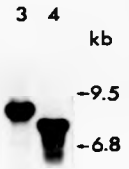
Table 6.2 Transformation frequencies of *Synechococcus* R2-SPc with TnphoA containing vectors

Plasmid	Transformants $\mu g\ DNA^{-1}$ Selection	
	Ap^r	Km^r
pUC105	10^5	zero
5.01	10^2-10^3	10^1-10^2 but unstable
9.01	zero	10^5

Insert 5.01 transformed *Synechococcus* R2 to Ap^r, but selection for Km^r produced very small colonies, which when restreaked on Allen's agar + 25 $\mu g\ ml^{-1}$ kanamycin failed to grow. Insert 9.01 produced stable Km^r transformants, but these were all Ap^s - reflecting the nature of the *bla-phoA* hybrid, mentioned above. Total DNA was isolated from Ap^r insert 5.01 containing cells, and Km^r insert 9.01 transformants. Southern hybridisation with a 1.3 kb *Xho*I-*Eco*RI internal *phoA* fragment, showed that no Tn*phoA*-homologous DNA was present in the cells which did not stably express Km^r - see Figure 6.4. Earlier experiments showed that *Synechococcus* R2 chromosomal DNA did not hybridize with *E. coli phoA* under stringent conditions - see section 5.3.3. The instability of

Figure 6.4 Detection of *phoA* sequences in *Synechococcus* R2-SPc by Southern blotting analysis

Filters were probed with a 1.3 kb *Xho*I-*Eco*RI internal *phoA* fragment from Tn*phoA*. Hybridisation and washing conditions were as described in Figure 5.4. Lane 1 - *Xho*I cut total DNA from *Synechococcus* R2-SPc containing plasmid 5.01; lane 2 - *Bam*HI cut total DNA from *Synechococcus* R2-SPc containing plasmid 5.01; lane 3 - *Xho*I cut total DNA from *Synechococcus* R2-SPc containing plasmid 9.01; lane 4 - *Bam*HI cut total DNA from *Synechococcus* R2-SPc containing plasmid 9.01.



Tn*phoA* in plasmid 5.01 correlates well with the proposed position of the cyanobacterial replication origin of this vector. Gendel *et al.* (1983), and work described here (see also section 7.3.1) showed that a 4.5 kb *Xho*I-*Bam*HI fragment was essential for successful plasmid pUC105 transformation and replication. Tn*phoA* in plasmid 5.01 maps within this *Xho*I-*Bam*HI fragment. Recently, Gendel (1987) showed that plasmids with Tn*5* inserts distributed throughout this 4.5 kb region were unstable, suggesting that the entire region may be structurally involved in replication. Tn*phoA* in plasmid 9.01 is stably maintained in *Synechococcus* R2 when selecting for the transposon marker, and here the transposon insert occurs in DNA shown not to be necessary for plasmid replication.

Synechococcus R2 cells containing plasmid 9.01 were grown in medium lacking endogenous chelator ± kanamycin 25 $\mu g\ ml^{-1}$, for 3-4 days, before penicillin enrichment. Surviving cells were plated onto normal medium ± kanamycin 25 $\mu g\ ml^{-1}$, but after 4-5 days incubation in the light at 30°C no colonies had appeared.

The ability of Tn*phoA* to transpose into the cyanobacterial chromosome might then, be questioned. Plasmid 5.01 is effectively acting as a suicide vector in *Synechococcus* R2-SPc. Assuming the plasmid can enter the cell, and since plasmid 5.01 cannot replicate in *Synechococcus* R2, the only means of maintaining Tn*phoA* is through its transposition into the *Synechococcus* chromosome. Southern hybridization which detected no homologous Tn*phoA* sequences in chromosomal DNA from Ap^R plasmid 5.01-containing cells, suggests that transposition has not occurred.

An alternative means of mutagenesis using Tn*phoA* could be to combine the advantages of transposon mutagenesis in *E. coli* with the high levels of recombination found in *Synechococcus* R2. Thus, a cyanobacterial gene library could be constructed in a suitable *E. coli* plasmid, e.g. pACYC184 - which lacks a gene product encoding an exported polypeptide - and mutagenised *in vivo* using Tn*phoA*. The mutated library could then be isolated and used to transform *Synechococcus* R2, selecting for the transposon marker.

6.3.2 Transposon mutagenesis of cyanobacteria using Tn5 and Tn1 derivatives

The inheritance of the suicide plasmids pSUP2021, RP4 :: Tn5, pDS4101 (Tn1-containing) and RP4 (Tn1-containing) was studied in *Synechococcus* R2. In addition, pSUP2021 was mobilised into *Synechococcus* sp. and *Nostoc* sp. MAC. pSUP2021 and pDS4101 required the S17.1 donor strain of *E. coli* to allow mobilization of these plasmids into the recipient cyanobacteria. Conjugations were performed as described in section 6.2.3, and transconjugants were screened for resistance markers carried by the various transposons.

As shown in Table 6.3, pSUP2021 was transferred most efficiently to *Synechococcus* sp., with a frequency 10^3-10^4 higher than for *Synechococcus* R2. No transconjugants were observed arising from transfer of pSUP2021 into *Nostoc* sp. MAC. RP4 :: Tn5, pDS4101 or RP4 failed to be conjugated into *Synechococcus* R2. This would suggest that these plasmids fail to replicate in the cyanobacterium, and that the transposon is not maintained - whether this be due to an inability to transpose, or poor antibiotic expression is not known. No spontaneous

Km^r cells were observed in control experiments.

Synechococcus R2 transconjugants arising from transfer of pSUP2021 were of two types: i) very small colonies, which did not grow when restreaked onto selective medium (kanamycin 25 $\mu g\ ml^{-1}$), and ii) colonies which appeared normal and grew on solid media and in liquid at 25 $\mu g\ ml^{-1}$ kanamycin, but died after repeated subculture. These transiently Km^r cells were Ap^s and Cm^s (pSUP2021 confers Ap^r and Cm^r, as well as Tn5-encoded Km^r). The ability of Tn5 to confer streptomycin resistance in *Synechococcus* R2 was tested by plating cells onto medium containing 10 $\mu g\ ml^{-1}$ streptomycin. No growth was seen at this level of antibiotic. Gendel (1987) showed that *Synechococcus* R2 containing Tn5 stably maintained on a shuttle vector was able to grown on medium containing 2 and 5 $\mu g\ ml^{-1}$ streptomycin, but not on that containing 10 $\mu g\ ml^{-1}$ - suggesting poor expression of this antibiotic resistance gene.

The instability of the Km^r phenotype in *Synechococcus* R2 transconjugants arising from transfer of pSUP2021, might be due to the unstable nature of transposed Tn5 inserts in the cyanobacterial chromosome. To assess whether Tn5 could be stably maintained in a chromosomal location, pSUP2021 was mobilised into *Synechococcus* R2 containing pBR325 integrated into the chromosome (see section 5.3.2). pSUP2021 contains pBR325 sequences and so homologous recombination events, followed by selection for Km^r, carried by the transposon allowed Tn5 to integrate into the chromosome. Km^r transcongugants occurred at a frequency of 10^{-6}-10^{-7}. Southern hybridization (using 2.38 kb and 2.35 kb *Xho*I Tn5 internal fragments as a probe) of total DNA isolated from ten individual

Km^r transconjugants and cut with *Eco*RI showed the presence of homologous Tn5 sequences (see fig. 6.5). Large hybridising fragments were obtained, presumably because Tn5 contains no sites for *Eco*RI and pBR325 only one site, and also *Eco*RI cuts *Synechococcus* R2 chromosomal DNA infrequently. pBR325 homologous DNA sequences were also identified in chromosomal DNA from transconjugants (data not shown). Transconjugants were stable upon repeated subculture into selective medium containing 25 $\mu g\ ml^{-1}$ kanamycin. This implies that Tn5 can be maintained within the cyanobacterial chromosome, and suggests that the unstable nature of transconjugants containing pSUP2021 in *Synechococcus* R2 more probably reflects the inefficiency of the transposition event.

In contrast to *Synechococcus* R2, *Synechococcus* sp. transconjugants arising from transfer of pSUP2021 were stable upon continued kanamycin selection, and were resistant up to 300 $\mu g\ ml^{-1}$ kanamycin in liquid medium. However, all colonies appeared normal and no filamentous or pigment mutants were observed. Southern hybridization (using i) 2.38 kb and 2.35 kb *Xho*I Tn5 internal fragments, and ii) a 1.25 kb *Eco*RI-*Hind*III fragment from pBR325 containing the Cm^r gene, as probes) of total DNA isolated from 10 individual transconjugants, and cut with *Eco*RI, showed the presence of homologous Tn5 sequences in all transconjugants (see Fig. 6.6). Plasmid DNA sequences were present in only 3 of these, though there was a high level of background hybridisation (see Fig. 6.6). These results suggest that transposition of Tn5 into *Synechococcus* sp. occurs, as well as the integration of the whole plasmid into the chromosome - perhaps by some illegitimate recombination event. The absence of transformants when total DNA from a mixture of transconjugants was transformed into *E. coli* strain MC1061 would suggest

Table 6.3 Conjugation frequencies of various suicide vectors into *Synechococcus* R2, *Synechococcus* sp. or *Nostoc* sp. MAC

Strain	Plasmid	Conjugation frequency Transconjugants per 10^9 cells mated
Synechococcus R2	pSUP2021	10^{-7}-10^{-8}
	RP4::Tn5	zero
	pDS4101	zero
	RP4	zero
Synechococcus sp.	pSUP2021	10^{-3}-10^{-4}
Nostoc sp. MAC	pSUP2021	zero

Figure 6.5 Detection of Tn5 and pBR325 sequences in *Synechococcus* R2-SPc by Southern blotting analysis

Filters were probed with a) 2.38 kb and 2.35 kb *Xho*I Tn5 internal fragments - lanes 1-14; and b) *Pst*I cut pBR325 - lanes 15-24. Hybridisation and washing conditions have been described previously (Figure 5.4). Lanes 1-9 - *Eco*RI cut total DNA from *Synechococcus* R2-SPc chromosomal DNA::pBR325::pSUP2021; 9 individual transconjugants; lanes 10-14 - *Eco*RI, *Hin*dIII, *Bam*HI, *Xho*I and *Eco*RV cut, respectively, total DNA from a single *Synechococcus* R2-SPc chromsomal DNA::pBR325::pSUP2021 transconjugant; lanes 15-18 *Pst*I, *Xho*I, *Eco*RI, *Xba*I cut, respectively, total DNA from a single *Synechococcus* R2-SPc chromosomal DNA::pBR325 derivative; lanes 19 and 20 *Eco*RI cut total DNA from *Synechococcus* R2; lanes 21-24 - *Hin*dIII, *Bam*HI, *Eco*RV, *Xho*I cut, respectively, total DNA from *Synechococcus* R2-SPc chromosomal DNA::pBR325::pSUP2021 transconjugants.

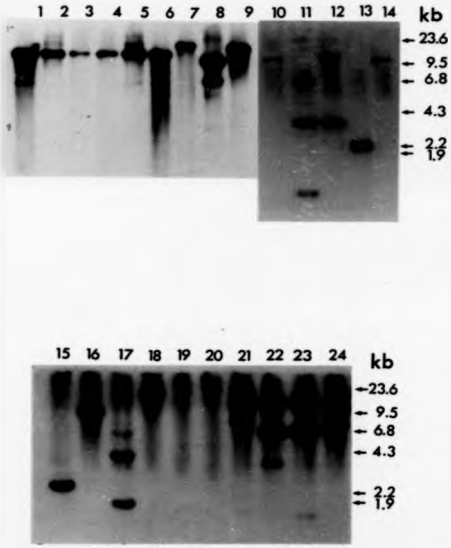
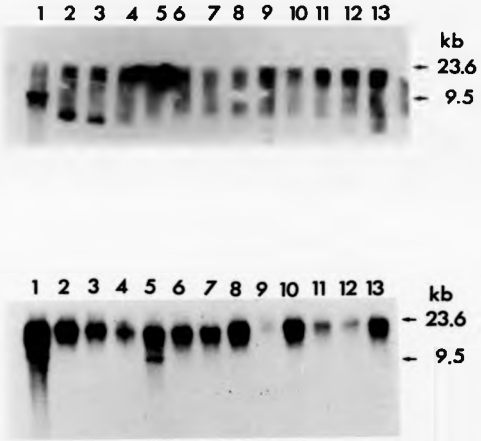


Figure 6.6 Detection of Tn5 and pSUP2021 sequences in *Synechococcus* sp. by Southern blotting

Filters were probed with a) 2.38 and 2.35 kb *Xho*I Tn5 internal fragments (bottom) and b) 1.25 kb EcoRI-HindIII fragment from pBR325 (top). Filters were hybridised at 65°C in 5 x SSPE, 5 x Denhardts, 0.1% SDS, and washed at 77°C in 0.1 x SSPE, 0.1% SDS. Lanes 1-13 contain *Eco*RI cut total DNA from *Synechococcus* sp. pSUP2021 individual transconjugants.



that pSUP2021 was not being maintained extra-chromosomally in *Synechococcus* sp.

Whilst *Synechococcus* sp. appears the most promising strain for sucessful transposon mutagenesis using Tn5, the lack of mutants following growth in liquid medium - which allows both segregation of a mutant phenotype, and, in our hands, removes any *E. coli* contamination following conjugation - suggests there is an inherant problem in this approach. One of the difficulties concerns the availability of suitable selection procedures - see section 6.3.4. Moreover, the possession of multiple genomes by cyanobacteria (see Herdman, *et al* 1979) is a major obstacle if transposon mutagenesis is to succeed.

Plasmid pRI46-containing Tn901, failed to transform *Synechococcus* R2 or *Synechococcus* sp. A Tn901-containing plasmid has previously been successfully used to produce a mutation in a gene involved in methionine biosynthesis (Tandeau de Marsac *et al.*, 1982).

6.3.3 Selection for chlorate resistance

Selection for resistance to chlorate, a technique used to isolate and characterize nitrate reductase mutants in bacteria, has been reported for a few heterocystous species in cyanobacteria (see Singh and Sonia, 1977). This positive selection for chlorate resistance, should have provided an ideal screening procedure for scoring mutants. However, using a final chlorate concentration of 2% in agar plates containing 20 mM nitrate and 0.1 mM ammonium - a lethal dose for *Synechococcus* PCC6301 (Lightfoot, 1985) - background growth was observed on plates containing as few as 10^4 colonies. (It was previously shown that toxicity was

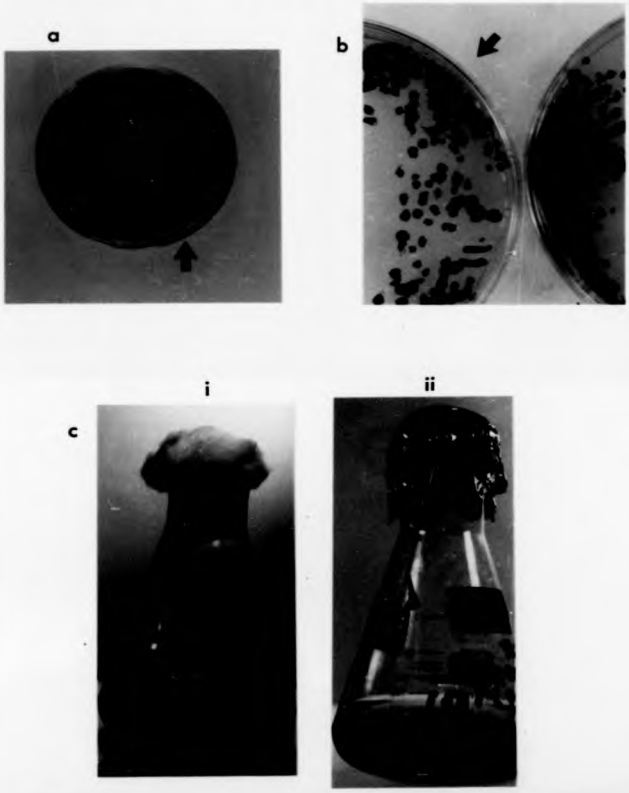
plating density dependent). The use of this selection procedure was thus discontinued.

6.3.4 Chemical mutagenesis of *Synechococcus* R2 and *Nostoc* sp. MAC

Synechococcus R2 and *Nostoc* sp. MAC were treated with NTG and cells transferred to selective medium lacking added chelator (see section 6.2.4). After penicillin enrichment (section 6.2.5) cells were plated onto normal medium. Approximately 5-10,000 colonies of both *Nostoc* sp. MAC and *Synechococcus* R2 appeared, which included two pigment mutants of *Nostoc* sp. MAC and one of *Synechococcus* R2 (see fig. 6.7). The pigment mutant of *Synechococcus* R2 appears to have decreased levels of phycocyanin, similar to the mutants of *Synechococcus* PCC6301 described by Yamanaka and Glazer (1981), which contained only 35% and 50% of the wild type level of phycocyanin. The yellow-green appearance of this mutant was accentuated under iron limitation (see fig. 6.7), which is consistent with induced phycocyanin degradation under such conditions. Preliminary characterization of these, and other pigment mutants of *Nostoc* sp. MAC (obtained by R. Kennedy, this laboratory) is underway.

Colonies surviving after penicillin enrichment were patched onto solid medium with and without added chelator, to screen for mutants defective in iron uptake. Of the 10% of the surviving colonies screened, all grew normally on chelator-deficient medium. This inability to obtain mutants defective in iron acquisition in *Synechococcus* R2 and *Nostoc* sp. MAC may, i) reflect the inadequacy of the screening procedure, or ii) mean that no mutants were obtained. This might imply that such a mutation is a lethal event. It is possible that the selection procedure is at

Figure 6.7 Pigment mutants of a) *Synechococcus* R2, and b) *Nostoc* sp. MAC after NTG mutagenesis and enrichment in chelator deficient medium.
Arrow indicates wild type strain. c) Growth of the *Synechococcus* R2 pigment mutant in chelator-deficient medium (i), compared to the wild type (ii).



fault. Although agarose was used as the solid support instead of agar, to reduce iron contamination, there may be enough exogenous iron remaining to permit growth. Perhaps a more stringent screening procedure would be to use a strong chelator such as EDDA, contained within the chelator-deficient medium. This may prove useful in the future.

6.4 Conclusion

The ability of Tn5 to transpose in cyanobacteria appears to be strain dependent. In *Synechococcus* R2, the transposon was stably maintained only when Tn5 was introduced into the cell by homologous recombination via plasmid vector sequences - transposition *per se* did not occur. However, *Synechococcus* sp. allows 'transposition' of Tn5 - as demonstrated by Southern hybridization - but this is not enough to produce a mutant phenotype. In this latter strain, it may be that more stringent selection procedures will provide more positive results, or perhaps the treatment of cyanobacterial cells with chemicals capable of reducing the chromosome number per cell, such as 2-phenylethanol (J. Reaston, unpublished observations).

A parallel study using Tn*phoA*, a transposon which can enrich for extracellular enzyme mutants - in *Erwinia carotovora* Tn*phoA* offers a 6-fold enrichment over Tn5 (1.7% compared to 0.3% for Tn5) for protein export mutants (Hinton and Salmond, 1987) - showed that Tn*phoA* could be stably maintained in a cyanobacterial shuttle vector, but no evidance of transposition was observed. An alternative approach to obtaining transposon mutants, using the efficient mutagenesis regime existing for

E. coli and the homologous recombination system for *Synechococcus* R2, has been proposed (see section 6.3.1). Although more laborious, this could provide the only useful way of obtaining genetically engineered mutants in cyanobacteria.

Chapter 7

The Construction of Promoter Probes for use in Studying Gene Expression in Cyanobacteria

7.1 Introduction

The dramatic advance in bacterial molecular genetics over the last decade has led to many interesting new developments, none more so than the gene fusion technology associated with the study of a wide variety of biological problems (for review see Silhavy and Beckwith, 1985).

We have set out here to modify and develop vectors for use in cyanobacteria, that use expression of an assayable gene product, such as β-galactosidase or neomycin phosphotransferase, put under the control of another gene of interest and in this way used as a tag to follow the expression of that latter gene. Such constructs permit assay for promoter activity and hence one can directly investigate the transcriptional control of specific gene promoters under varying environmental stimuli. In addition, these gene fusions allow DNA sequences not normally present in *E. coli* to be examined for the presence of sequences capable of serving as promoters in *E. coli*., and DNA fragments of particular interest can be isolated using physical techniques without relying on any genetic properties.

The ability of β-galactosidase to be expressed from both the *Synechococcus* R2 chromosome, or on a multicopy plasmid (section 5.3.2) in cyanobacteria, will allow us to utilise the many techniques available for the study of *lac* fusions and this is useful especially since the *lac* operon is one of the most extensively studied genetic systems.

A general technique for studying *lacZ* fusions to genes that specify exported proteins has been described (Palva and Silhavy, 1984), and we will consider its usefulness in this area, and describe fusions to other

genes for analysis of protein export signals. The use of Tn*phoA*, and *pho*A fusions has already been described (see Section 6.1.6).

7.1.2 Development and use of lac fusions in the study of biological problems

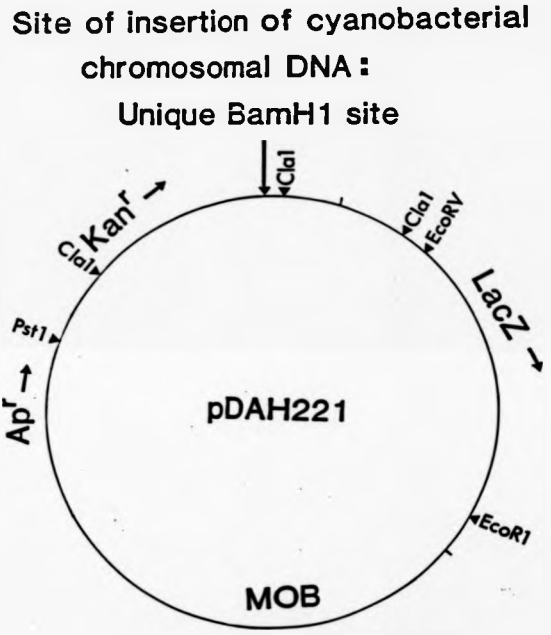
In developing a general method for constructing *lac* fusions, the *lac* genes must first be moved to a chromosomal location that is closely linked to the target gene and they must be in the same transcriptional orientation. Secondly, a genetic rearrangement that creates the appropriate novel fusion joint must be selected. Casadaban and Chou (1984) constructed a defective phage - Mu d2 (Ap lac), which when inserted into a target gene in the correct orientation generated a hybrid gene. This then encoded a hybrid protein if the reading frame was correct. Such fusions, however, are unstable and these *in vivo lac* fusions generally cannot be adapted directly for use in other organisms. Since then, transposons have been used which both extends the use of lac fusions *in vivo* to other organisms, and, depending on the transposon, will increase their stability. Youngman *et al.* (1985) used transposon Tn917 for the construction of *lac* fusions in *Bacillus subtilis*, whilst Kroos and Kaiser (1984) have constructed a Tn5 derivative that creates *lac* fusions upon insertion into *Myxococcus xanthus*. The lack of an efficient transposable element for use in cyanobacteria (see Chapter 6) has meant that such *in vivo* studies have not yet been possible in these organisms.

The advent of recombinant DNA technology, however, has provided a powerful methodology for the construction of novel DNA joints *in vitro*. The aim of such constructions, as we have seen above, was to devise a

procedure in which creation of the desired novel joint caused activation of a particular enzymatic activity. As regards lacZ this required the physical separation of the *lac*Z sequences required to specify a functional enzyme from the signals that direct transcription and translation initiation. The standard vector for constructing lacZ fusions *in vitro* contains a lacZ gene truncated at the 5' end and at a site before codon 26 (to separate the essential *lac*Z coding sequences from these expression signals). Consequently, the gene is missing a promoter, a ribosome binding site, and an ATG initiation codon. At the point of the truncation, synthetic DNA sequences containing multiple restriction enzyme cleavage sites have been inserted (see Fig. 7.1). Thus if a 5' coding sequence containing the required expression signal is cloned into one of the restriction enzyme cleavage sites so that transcription and translation are restored across *lac*Z, then a hybrid protein with β-galactosidase activity is produced. The efficiency or strength of the promoter sequences carried on this inserted DNA then determines the level of β-galactosidase activity, assuming that it is free of influences that are not promoter-specific, such as those that are related to mRNA translation and mRNA stability.

The vectors described above have been used for the construction of gene fusions that specify hybrid proteins. Vectors that can be used for the construction of *operon* fusions also exist. These have become less popular, since i) in heterologous systems the translation initiation signals of *lac*Z are often not recognised by the organism under study and ii) plasmid vectors designed for the construction of operon fusions are difficult to use.

Figure 7.1 Restriction map of pDAH221, a mobilizable lacZ promoter probe



7.1.3 Use of lacZ fusions in studying protein export

Palva and Silhavy (1984) devised a general, one-step technique for the isolation of *E. coli* strains in which the gene coding for an exported protein was fused to *lacZ*. These fusions specified a hybrid protein comprised of an NH_2-terminal portion of the exported protein and a large functional COOH-terminal portion of β-galactosidase. They were constructed with a derivative of the Mu dII (lac, Ap) phage (Casadaban and Chou, 1984) mentioned earlier. The lethality often associated with the high-level expression of such hybrid genes, e.g. *lamB-lacZ* and *malE-lacZ* fusions (Bankaitis *et al.*, 1985), was overcome by inserting an early *lacZ* nonsense mutation onto this phage. The use of strains carrying a temperature-sensitive nonsense suppressor, then allowed controlled expression of the full length hybrid protein by varying the growth temperature. The utility of this technique was demonstrated by isolating a series of gene fusions to *ompA*, coding for a major outer membrane protein.

The over-production lethality mentioned above, is caused by the cells abortive attempts to export the β-galactosidase portion of the hybrid protein. The hybrid protein apparently contains sequences that jam the cell export machinery, thus hindering normal protein export. When expression of the hybrid gene is high, such inhibition of normal export is lethal.

Work using *malE-lacZ* fusions and *lamB-lacZ* fusions has provided considerable insight into the protein export pathway in *E. coli*. Ito *et al.* (1981) showed that secretion of a *malE-lacZ* fusion protein was initiated by the signal peptide attached to the N-terminus of the

maltose-binding protein sequence, but was not completed, presumably because the β-galactosidase moiety of the hybrid protein interfered with the passage of the polypeptide through the cytoplasmic membrane. Under these conditions, when the protein became stuck in the cytoplasmic membrane, periplasmic proteins including maltose-binding protein and alkaline phosphatase, and the major outer membrane proteins, including OmpF and OmpA, were synthesized as precursor forms with unprocessed signal sequences. It seems that specific sites in the cytoplasmic membrane become progressively occupied by the hybrid protein, resulting in an inhibition of normal localization and processing of periplasmic and outer membrane proteins. Such results suggest that most of the periplasmic and outer membrane proteins share a common step in localization before the polypeptide becomes accessible to the processing enzyme. Herrero *et al.* (1982) have confirmed this phenomena, and also showed the accumulation of one outer membrane protein, but at least two inner membrane proteins as unprocessed forms.

Gene fusions have also shown the essential role of the signal sequence in the critical steps of protein secretion and that several regions within the mature maltose binding protein and lamB protein have an important role in protein export (see Bankaitis *et al.*, 1985). We have already seen that it is not sufficient to attach an exported protein to a non-exported protein to ensure its export through the cytoplasm (Pugsley and Cole, 1986). There are a number of sites within the β-galactosidase polypeptide that are not compatible with its passage through the cytoplasmic membrane. However, we should qualify this. Class IV *lamB-lacZ* fusions (including an intact signal peptide and approximately the first 240 residues of the mature LamB protein) are

incorporated into the outer membrane at an efficiency of 85-90%. In addition, lkyB-lacZ hybrid protein fusions were partially extracellular and envelope associated, the first known example of a spontaneous release by *E. coli* of a hybrid β-galactosidase molecule into the extracellular medium (Lazzaroni and Portalier, 1985). Such *E. coli* periplasmic-leaky (*lkyB*) mutants excrete alkaline phosphatase and β-lactamase into the extracellular medium and are altered in their outer membrane protein content. Thus the use of β-galactosidase fusions in studying the later stages of protein export is controversial. With this in mind the construction of new gene fusions has been initiated (Section 7.1.4).

The work described above has primarily been of use only in *E. coli* - where genetic techniques are widely advanced. Nonetheless, there is no reason to suggest against the use of such gene fusions in studying protein export in more diverse organisms such as cyanobacteria.

7.1.4 Secretion vector systems

Broome-Smith and Spratt (1986) have described the construction of translational fusions to TEM β-lactamase for the analysis of protein export signals. This system might be preferable to the use of alkaline phosphatase fusions (Hoffman and Wright, 1985; Manoil and Beckwith, 1985; Section 6.1.6), since in the latter system only those fusions that translocate alkaline phosphatase across the cytoplasmic membrane are identified. Using β-lactamase it is possible to identify all in-frame fusions (by the ability of transformants to grow when plated at high inocula on agar containing ampicillin), and subsequently to determine whether or not the β-lactamase moiety of the fusion protein

has been translocated (by the ability, or inability of the fusion protein to protect single cells against lysis by ampicillin). Edelman *et al.* (1987) used this fusion vector to investigate the organization of penicillin-binding protein 1B in the cytoplasmic membrane of *E. coli*. A similar translational fusion vector was constructed using the plasmid encoded β-lactamase of *Staphylococcus aureus* (Wang *et al.*, 1987). To go one step further, Smith *et al.* (1987), constructed vectors containing genes coding for two extracellular proteins - the α-amylase from *Bacillus licheniformis*, and the β-lactamase from *E. coli* - which lacked part of, or the entire signal sequence. Since signal sequences are functionally interchangeable, cloning in the correct reading frame of DNA fragments coding for a promoter, ribosomal binding site, start codon, and signal sequence will result in the synthesis and export of the protein.

7.1.5 Prokaryotic promoter - probe vectors

To date, 168 promoters of *E. coli* have been reported and these show a variable amount of homology to a previously formulated consensus sequence (Pribnow, 1975). The DNA sequences of all promoters contain signals that are recognised by RNA polymerase, as well as containing information that determines its efficiency. The efficiency of a promoter can be modulated in many ways (de Boer and Shephard, 1983). Systems developed for this purpose have been described for various bacteria, by assaying the expression of a single gene, e.g. in *E. coli* using *galK*, Cm^R or tet^R; in *Caulobacter crescentus* using neomycin phosphotransferase II; in *Bacillus* spp. using luciferase, and in filamentous cyanobacteria using the luciferases from *Vibrio harveyi* and *Vibrio fischeri* (see, for example, Schmetterer *et al.*, 1986). Promoter-

probe vectors for the analysis of divergently arranged promoters (which direct transcription bidirectionally from closely spaced sites) have also been described (Schneider and Back, 1986).

The use of luciferase genes as a promoter probe in filamentous cyanobacteria allows sufficient sensitivity to monitor the transcription of single genes in single cells of a cyanobacterial filament (Elhai and Wolk, 1987). Luciferase catalyses a light-producing reaction;

$$\text{alkyl aldehyde} + FMNH_2 + O_2 \xrightarrow{\text{luciferase}} \text{alkanoic acid} + FMN + H_2O + \text{Light}$$

FMN is flavin mononucleotide and $FMNH_2$ reduced flavin mononucleotide.

Using a shuttle vector pRL488, that facilitates the cloning of DNA upstream from the genes encoding luciferase from *Vibrio fischeri*, the strengths of several promoters in *Anabaena* PCC7120 have been investigated (Elhai and Wolk, 1987). The rubisco promoter was very strong, as was the *tac* promoter (-35 promoter of *trp* and -10 from *lac* UV5) - which could be regulated by the *lac* repressor, and IPTG increased activity 3-4 fold. The *nif*KDH and *gln*A promoters were found to be relatively weak and the neomycin phosphotransferase promoter from Tn5 had little or no activity. The fact that light emission can be observed from single cells in these filamentous cyanobacteria using such promoter fusions however, should provide interesting information on the control of gene activity during differentiation, providing problems with measurement variability and the ability to distinguish low promoter activity, and hence low luciferase expression, from background can be overcome.

7.2 Materials and Methods

All molecular biological techniques and transformation procedures were performed as described in Chapter 2, whilst conjugation experiments were carried out as stated in Chapter 5. ONPG and MUG assays, and *E. coli* cell fractionation procedures have been described previously (Section 5.2.1 and Section 3.2.2 respectively). Protein estimation was by the Biorad Method (Section 2.7.3).

7.3 Results and Discussion

7.3.1 Construction of lacZ promoter probes for use *Synechococcus* R2 PCC7942

The suitability of pDAH221 (see Fig. 7.1) - a kind gift of D. A. Hodgson, this laboratory - for assessing promoter activity, led us to construct various libraries of *Synechococcus* R2 chromosomal DNA in this vector. pDAH221 is incapable of independent replication in cyanobacteria, but contains a unique BamHI site upstream of the promoterless *lacZ* gene for insertion of cyanobacterial DNA. This vector also contains *mob* functions which allows conjugal transfer of this plasmid into cyanobacteria from an appropriate *E. coli* host, e.g. S17.1.

*Bam*HI digested pDAH221 DNA was phosphatased using calf intestinal phosphatase (Section 2.9.4) to prevent vector self-religation, and *Synechococcus* R2 chromosomal DNA was partially digested with *Sau*3A to produce 0.5 kb and 4 kb DNA fragments as inserts in library constructions. Transformation of *E. coli* strains DH1, MC1061 (*lac* X74 deletion) and S17.1 (containing RP4 integrated into the chromosome) with ligated vector and chromosomal DNA, using the higher efficiency SOB

transformation method, produced partial gene libraries of *Synechococcus* R2 chromosomal DNA in pDAH221. The presence of inserts was confirmed by cutting small-scale plasmid preparations of transformants with *Bam*HI. Cloning of *Sau*3A inserts into a *Bam*HI site destroys the latter site. 19/20 transformants failed to cut with *Bam*HI.

Considerable variation in transformation frequency was observed between *E. coli* strains (see Table 7.1) and thus the choice of *E. coli* host is an important consideration if a complete gene library is required in this vector. (Ligation mixtures transformed the respective *E. coli* strains with a 100- 1000 fold reduced frequency compared to pDAH221).

The partial gene libraries of *Synechococcus* R2 chromosomal DNA in pDAH221, purified on caesium chloride gradients, were used to transform *Synechococcus* R2 and *Synechococcus* R2-SPc. Transformation frequencies were poor (see Table 7.2), and transformants were observed i) only under Km selection (25 $\mu g\ ml^{-1}$) but not Ap selection (0.5 $\mu g\ ml^{-1}$), and ii) only with 4 kb *Synechococcus* R2 chromosomal DNA inserts, but not 0.5 kb inserts. The size of pDAH221 (11.1 kb) may be partly responsible for the low transformation frequencies, since Kolowsky *et al.* (1984) observed that the probability of integration decreased by half for each increase of 2-3 kb of foreign DNA. The Km^r transformants were Ap^s, and repeated transfer of transformants in selective media resulted in the loss of kanamycin resistance, suggesting their inherant instability. No detectable β-galactosidase activity was observed, as assessed by spraying plates with MUG (10 mg/ml).

Table 7.1 Transformation frequencies of plasmids pDAH221 and pBR322 in various *E. coli* strains

	Transformants $\mu g\ DNA^{-1}$		
	E. coli strain		
plasmid	DH1	S17.1	MC1061
pDAH221	2×10^4	8.0×10^4	1.23×10^6
pBR322	5×10^5	8.8×10^6	2.56×10^7

Table 7.2 Cyanobacterial shuttle vector, integrative vectors and promoter probe vector transformation frequencies

Strain	Plasmid/selection	Transformants $\mu g\ DNA^{-1}$
Synechococcus R2	pUC105 Cm^r	10^3-10^5
	pLAH4 Cm^r	10^3-10^3
	pDAH221::R2 4kb chromosomal DNA Km^r	10^1-10^2
	pLACPB1::R2 0.5 kb & 4 kb chromosomal DNA Cm^r	10^2-10^3
	pKIC-7::4kb chromosomal DNA Km^r	10^1-10^2
Synechococcus R2-SPc	pUC105 Cm^r	10^5
	pUC105 XhoI-SalI deletion Cm^r	10^4
	pUC105 HindIII deletion Cm^r	zero
	pLAH4 Cm^r	10^4-10^5
	pKIC-7::4kb chromosomal DNA Km^r	10^2-10^3
	pLACPB1::0.5kb & 4kb chromosomal DNA Cm^r	10^5-10^6
Synechococcus sp.	pUC105	10^6
	pLAH4	10^2-10^3
	pKIC-7::4kb R2 chromosomal DNA	10^1-10^2
	pLACPB1::4kb R2 chromosomal DNA	10^5-10^6

Southern blotting of chromosomal DNA from these transformants, and using i) the 7.2 kb *Bam*HI-*Eco*RI fragment of pDAH221 containing Km^r and Ap^r genes, and ii) the complementary 3.95 kb BamHI-EcoRI fragment of pDAH221 containing the *lac* gene as probes, showed the presence of homologous hybridising DNA sequences in transformants probed with the former DNA fragment, but no hybridization with the latter probe (data not shown). The absence of *lacZ* gene sequences in transformants explains the lack of β-galactosidase activity, and suggests the deletion of donor DNA containing Ap^r and *lacZ* gene sequences upon integration into the cyanobacterial genome. Williams and Szalay (1983) showed foreign DNA linked to the ends of the cyanobacterial DNA fragment could integrate only in type II and III configurations. These transformants were less stable than type I transformants (see Section 5.1.6).

pDAH221 :: *Synechococcus* R2 chromosomal DNA libraries were conjugated into *Synechococcus* R2 using the *E. coli* strain S17.1. A conjugation frequency of 10^{-7}-10^{-8} was obtained for the 4 kb chromosomal library, i.e. 10^{1}-10^{2} transconjugants per 10^{9} cells mated, whilst the 0.5 kb library produced no transconjugants. Kanamycin selection was used routinely to avoid the problems associated with selection of Ap^r, i.e. release of β-lactamase from *E. coli* cells, conferring Ap^r on cyanobacterial cells. 46/46 Km^r transconjugants produced lacZ activity as assessed by MUG spraying, but there were no observable differences in lacZ activity between transconjugants. Although transconjugants were resistant to 120 μg/ml Km there was no detectable difference in lacZ activity with increasing Km^r, suggesting that readthrough from the Km^r gene was not responsible for lacZ activity.

The stability of these transconjugants is in contrast to the instability of respective transformants. This may correlate with the mode of transfer of DNA - conjugation involves the transfer of single-stranded DNA and a physical contact between cells, and this may produce more stable constructs than the uptake of naked DNA via transformation.

The failure of 0.5 kb fragments of *Synechococcus* R2 chromosomal DNA to integrate suggests that there is a lower size limit of DNA which is required for homologous recombination into the chromosome. The idea of using small chromosomal DNA inserts was to attempt the ectopic mutagenesis of *Synechococcus* R2 DNA. This procedure was described by Buzby *et al.* (1985) who used random *Sau*3A fragments of *Agmenellum quadruplicatum* PR-6 ligated to Tn*1* as a means of promoting recombination of the selectable ampicillin resistance gene with the chromosome at multiple sites. This would cause gene inactivation by a mechanism similar to that shown in Figure 5.5.

The low transformation/conjugation frequencies of the pDAH221 :: *Synechococcus* R2 chromosomal DNA libraries led us to construct an alternative lacZ promoter probe (Fig. 7.2), based on the shuttle vector pUC105 (Kuhlemeier *et al.*, 1981). The *Bam*H1 site in pUC105 was removed by filling in cut ends using the Klenow fragment of DNA polymerase, and the resulting vector (pUC105 Bam$^-$) cut with *Xho*I-*Sal*I. Deletion experiments with pUC105 showed that a *Xho*I-*Sal*I deletion did not affect transformation frequency (Table 7.2). This was in contrast to deletion of a 4 kb *Hind*III fragment from pUC105 which completely abolished replication of pUC105 in *Synechococcus* R2. This would agree with the proposal that the cyanobacterial replication origin of this plasmid is

contained within a 4.65 kb *Bam*HI-*Xho*I restriction fragment (Gendel, 1987). Insertion of a promoterless *lac*Z gene from pDAH216 (a gift of D. A. Hodgson, this laboratory) into the large *Xho*I-*Sal*I fragment of pUC105 Bam^- produced pLACPB1, which contains a unique *Bam*HI site for insertion of cyanobacterial chromosomal DNA. 0.5 kb and 4 kb *Synechococcus* R2 chromosomal DNA libraries were subsequently constructed in this vector, cloning partial *Sau*3A digested chromosomal DNA fragments into this unique *Bam*HI site.

Plasmid promoter probe vectors have not been widely used for studying gene fusions, because the background expression of an intact *lac*Z gene on a multicopy plasmid, even lacking a recognisable promoter, is apparently quite high (D. Hodgson, personal communication). Thus, it may be difficult to distinguish strains carrying the desired fusion from those carrying the parent plasmid. pLACPB1 possesses some endogenous LacZ activity (see Tables 7.3 and 7.4). This activity is negligible in *E. coli*, and in *Synechococcus* R2 is 10-20 fold reduced in the absence, compared to the presence, of inserted *Synechococcus* R2 chromosomal DNA fragments. The construction of vectors which have transcription termination signals both upstream of *lac*Z and the site of insertion of chromosomal DNA, and downstream of *lac*Z will reduce this problem further.

The expression in *E. coli* of various *Synechococcus* R2 chromosomal DNA :: *lac*Z gene fusions was determined in cytoplasmic, periplasmic and supernatant fractions of the cell using ONPG assays (see Table 7.3). Between transformants a wide variation in LacZ activity was observed, ranging from 1×10^{-2} Δ 420 min^{-1} mg $protein^{-1}$ to 720 Δ 420 min^{-1} mg

protein^{-1} in cell lysates. This activity can be correlated with the strength of cyanobacterial promoters in *E. coli*, though the level of expression is also proportional to the copy number of the plasmid in *E. coli*. pACYC184, the *E. coli* replicon in pLACPB1, is multicopy in *E. coli*. For comparison, chromosomal expression of lacZ in *E. coli* strain DH1 is 3.5 Δ 420 min^{-1} mg protein^{-1}. Over 90% of LacZ activity was cytoplasmically located, and this correlates well with the known cytoplasmic location of this protein. The minimal activity recorded in periplasmic and supernatant fractions may be a result of cell lysis. The negligible activity observed from two of the pLACPB1 :: 0.5 kb inserts is presumably a result of the absence of promoter sequences within these small chromosomal inserts.

These results suggest that cyanobacterial DNA sequences capable of serving as promoters in *E. coli* can be identified, and their strength ascertained using a simple enzyme assay (assuming that the plasmid copy number is similar in each case). This would allow DNA fragments of particular interest to be easily isolated using the wide variety of molecular biological techniques available in *E. coli*.

pLACPB1 :: 0.5 kb and 4 kb chromosomal DNA libraries were transformed into *Synechococus* R2, *Synechococcus* R2-SPc and *Synechococcus* sp. (the strain used for the transposon mutagenesis studies described in Chapter 6). Transformation frequencies (Table 7.2) were 10-100 fold reduced in *Synechococcus* R2 compared to the small plasmid cured strain. The latter strain, lacking the resident pUH24 plasmid, prevents possible loss of the cloned DNA through recombination events between the two plasmids, estimated to occur in up to 90% of the transformed population

Figure 7.2 Construction of pLACPB1: a LacZ promoter probe for use in Synechococcus R2

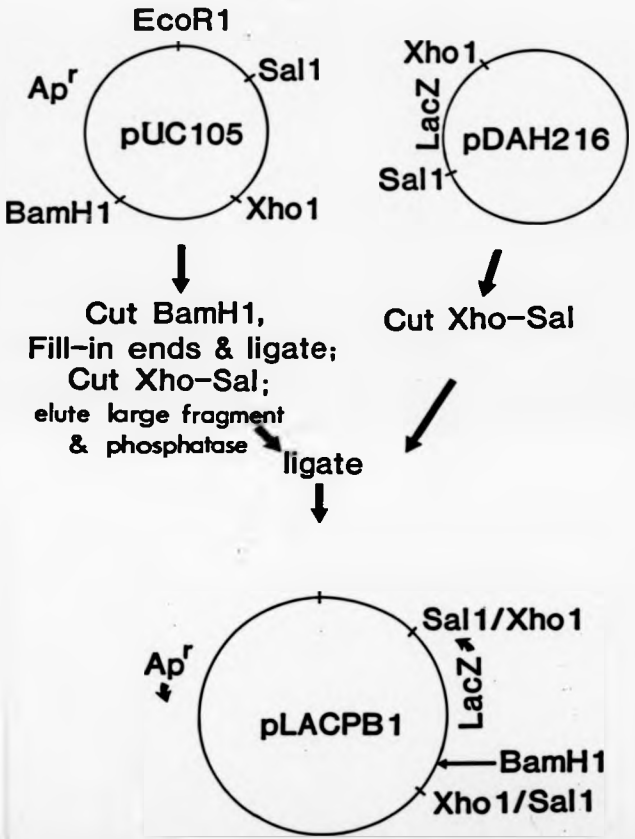


Table 7.3 ONPG β galactosidase assays in cytoplasmic, periplasmic and supernatant fractions of *E. coli* containing pLACPB1::*Synechococcus* R2 chromosomal DNA

Strain	β-galactosidase activity Δ 420 min^{-1} $mg\ protein^{-1}$		
	lysate	periplasm	supernatant
pLACPB1:: 0.5 kb	30.0 (91.4%)	2.13 (6.5%)	0.7 (2.1%)
chromosomal DNA	$1.1x10^{-2}$ (100%)	zero (0%)	zero (0%)
	$3.6x10^{-2}$ (90.8%)	$3.65x10^{-3}$ (9.3%)	zero (0%)
	9.83 (89.8%)	0.8125 (7.4%)	0.3 (2.7%)
	106.6 (92.8%)	6.09 (5.3%)	2.2 (1.9%)
pLACPB1:: 4 kb	49.5 (93.2%)	1.6 (3%)	2.04 (3.8%)
chromosomal DNA	720.45 (94.8%)	26.6 (3.5%)	12.48 (1.6%)
	12.625 (86.8%)	1.26 (8.7%)	0.66 (4.5%)
	6.12 (89.4%)	0.297 (4.3%)	0.428 (6.3%)
	57.24 (94.3%)	2.24 (3.7%)	1.22 (2.0%)
	8.19 (90.6%)	0.4 (4.4%)	0.45 (4.9%)
	38.2 (93.3%)	0.99 (2.4%)	1.74 (4.25%)
	9.4 (94.5%)	0.125 (2.7%)	0.13 (2.8%)
	11.0 (97.2%)	0.15 (1.3%)	0.165 (1.5%)
	52.18 (97.8%)	0.809 (1.5%)	0.366 (0.7%)
DH1	3.53 (89.7%)	0.252 (6.4%)	0.15 (3.8%)
pLACPB1	$6.9x10^{-2}$ (100%)	zero (0%)	zero (0%)

Table 7.4 Fluorimetric MUG assays of β-galactosidase activity in *Synechococcus* containing pLACPB1::*Synechococcus* chromosomal DNA

Plasmid	picomoles MUG hydrolysed ml^{-1} min^{-1} normalised for culture density*
pLACPB::4kb chromosomal DNA	1.49
	0.96
	1.92
	1.13
	2.15
	3.54
pTUC1	4.76
Synechococcus R2 chromosomal DNA ::pBR325::pTEBG3	1.61
pLACPB1 endogenous activity	0.20

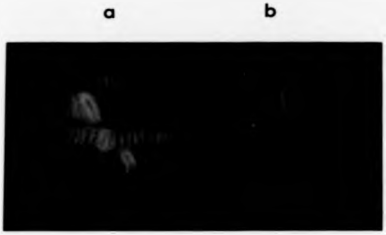
*one unit of specific activity is defined as one picomole of MUG hydrolysed per ml of culture sample per minute, normalized for culture density (OD_{750})

(Kuhlemeier *et al.*, 1981). In contrast to our results, however, Chauvat *et al.* (1983) showed that under optimal transformation conditions *Synechococcus* R2-SPc transformed consistently lower (about 30x) than the wild type strain. Such differences may be due to variation in strain handling, reflect curing procedures or more likely, result from the isolation of single colonies of these strains with greatly increased/decreased transformation capabilities.

The successful transformation of *Synechococcus* sp. with vectors based on *Synechococcus* R2 replicons or chromosomal DNAs, suggests this organism may be closely related to *Synechococcus* R2. Southern blotting experiments using the large subunit of rubisco (*rbc*L), glutamine synthetase (*gln*A) and allophycocyanin (*apc*) genes showed similar hybridising restriction fragments between *Anacystis nidulans* PCC6301 and *Synechococcus* sp. (M. R. K. Alley, personal communication) reinforcing the idea of a close relatedness between these strains. The ability of *Synechococcus* sp. to allow replication of *Synechococcus* R2 vectors and its potential use in mutagenesis studies, make it a choice organism for further study.

The expression of LacZ in *Synechococcus* R2 transformed with pLACPB1 :: chromosomal DNA libraries was investigated (see Table 7.4) using a fluorimetric MUG assay (section 5.2.1). The sensitivity of this method over ONPG assays allowed the detection of low LacZ activity in cyanobacterial cells. Expression of LacZ was higher from a supposed *E. coli* promoter (in pTUC1) than from cyanobacterial promoters (pLACPB1 libraries), both vectors based on the shuttle vector pUC105. β-galactosidase activity from a plasmid (pTUC1) versus a chromosomal

Figure 7.3 Differential expression of LacZ by *Synechococcus* R2 transformants containing pLACPB1. (a) iron-deficient medium. b) magnesium-deficient medium



location (*Synechococcus* R2 chromosomal DNA :: pBR325 :: pTEBG3) - 3-fold higher in the former - is given for comparison (Table 7.4 and see Chapter 5).

To assess the use of pLACPB1 in studying cyanobacterial gene expression under different environmental conditions, transformants (pLACPB1 :: *Synechococcus* R2 chromosomal DNA) were replica plated onto solid media containing agarose (Section 6.1) lacking any exogenous iron, or magnesium. Screening of such plates by spraying with MUG (see Fig. 7.3) suggested that iron inducible/repressible or magnesium inducible/ repressible genes may be detectable using such a gene fusion approach. Further work in this area should be fruitful.

7.3.2 Construction and use of a kanamycin promoter probe in cyanobacteria

Plasmid pKIC-7, a transformable kanamycin promoter probe, was constructed by ligating a *Bgl*II-*Sal*I fragment from Tn5 into *Bam*HI-*Sal*I digested pICI9-H. This contains a single *Bgl*II site for insertion of *Sau*3A digested chromosomal DNA. 0.5 kb and 4 kb *Sau*3A libraries of *Synechococcus* R2 chromosomal DNA were constructed in this vector, and transformed into *Synechococcus* R2 and *Synechococcus* R2-SPc. Transformants were selected at 25 $\mu g\ ml^{-1}$ kanamycin. Transformation frequencies (Table 7.2) were higher in the small plasmid cured strain, and 0.5 kb chromosomal DNA inserts failed to transform either wild type or plasmid cured *Synechococcus* R2 (see Section 7.3.1). Transformants were patched onto plates containing varying concentrations of kanamycin (25-400 $\mu g\ ml^{-1}$). All transformants were resistant to 100 $\mu g\ ml^{-1}$ kanamycin, and 19/29 grew at 150 $\mu g\ ml^{-1}$, 26/80 at 200 $\mu g\ ml^{-1}$, 8/80 at

300 $\mu g\ ml^{-1}$ and 1/80 at 400 $\mu g\ ml^{-1}$. These results suggest that such a promoter probe can be used to positively select for strong promoter activity via kanamycin resistance. The main drawback in its use is the necessity of having to replica plate large numbers of transformants to assess differences in promoter activity. In addition, although a sensitive method for the quantitative assay of neomycin phosphotransferase activity has been described (Reiss *et al.*, 1984) (the assay requires the electrophoretic separation of the enzyme from other interfering proteins and detection of its enzymatic activity by *in situ* phosphorylation of the antibiotic kanamycin), this is both inconvenient and time-consuming. Thus, the lacZ promoter probes described in Section 7.3.1 have largely superceded this vector for use in the unicellular cyanobacteria under investigation here.

pKIC-7 has since been modified for use as a conjugal promoter probe by ligating *Hind*III-*Sal*I digested pKIC-7 with *Hind*III-*Sal*I digested pSUP203 (M. R. K. Alley, this laboratory). This widens its use to filamentous cyanobacteria, and allows an investigation of the suitablity of such a vector in assessing differential gene expression in *Nostoc* sp. MAC.

7.4 Conclusion

The construction of *lacZ* and neomycin phosphotransferase promoter probes described in this Chapter, allows a powerful analysis of specific stimuli on cyanobacterial gene expression. Hence, the use of *lacZ* gene fusions in studying protein export for example, is now a realistic proposition in unicellular cyanobacteria. A preliminary examination of the application of *lacZ* gene fusion technology to iron and magnesium

limitation - effects which we have already seen affect protein export (Chapters 3 and 4) - suggests that we can be confident of future breakthroughs in this area. Moreover, the application of this technology to filamentous strains such as *Nostoc* sp. MAC allows analysis in a much more complex organism - an exciting prospect.

Concluding Remarks

The work described in this thesis has shown that cyanobacteria can be considered a major group of Gram-negative bacteria capable of protein secretion. Various pieces of evidence suggest this is a 'true' export process rather than some non-specific leakage of protein from the cell. However, the function of these polypeptides remains essentially cryptic. The photoautotrophic growth of these organisms may offer clues in this respect, and we have some evidence that implicates a nutrient-binding function. Certainly, this mode of nutrition is fundamentally different from the heterotrophic bacteria - which secrete a great variety of degradative enzymes - of which all our knowledge of protein secretion by Gram-negative bacteria derives.

Interest in the production of foreign proteins by bacteria has increased dramatically in the last few years, and with the world market for genetically engineered proteins in the near future predicted to be several billion dollars (see Nicaud *et al.*, 1986), the need for suitable microorganisms capable of secretion of foreign proteins becomes apparent. Whether cyanobacteria are suitable hosts in this respect is debatable, since although they are easily grown in large scale on cheap substrates, there is considerable competition from other bacteria with better characterised genetic systems, e.g. *Escherichia coli*, *Erwinia* sp. and from the Gram-positive bacilli capable of producing grams of secreted protein. Even so, the most suitable host for secretion of foreign proteins has yet to be found, and perhaps only greater understanding of the nature of the secretion process itself will allow us to fully exploit this system on an industrial scale. Further

progress in the development of a suitable transposon mutagenesis protocol, especially in the use of *Synechococcus* sp., and in the application of gene fusion technology may make cyanobacteria more suitable candidates.

Functional analyses of cyanobacterial outer membrane proteins suggest this class of exported protein is important in nutrient uptake, and in 'sensing' the external environment. Induction of specific polypeptides in the outer membrane under conditions of iron limitation is a widespread phenomenon among bacteria, but the presence of magnesium inducible polypeptides has not been heretofore reported. Further analysis of these induction processes might be directed towards i) characterizing the period of time, or the extracellular concentration of the limiting nutrient before induction occurs, ii) determining whether such polypeptides are a result of new protein synthesis and iii) assessing the effect of restoration of the limiting nutrient on derepression of these polypeptides. Such experiments would be assisted by radioactivaly labelling proteins using ^{35}S.

Whether such polypeptides are induced as a general stress response might be investigated, i) by construction of specific mutations in these outer membrane proteins by interposon mutagenesis, or ii) by assessing LacZ activity under various nutrient limitations. The first proposal requires the cloning of specific genes. The availability of 20-30 positive plaques, identified after screening a *Synechococcus* R2 λgt11 library with antisera to *Synechococcus* R2 cell walls, allows this approach. An immuno-positive λgt11 clone cross-reacting to this antibody, could be subcloned into an expression plasmid, and the

bacterial lysate, including plasmid-encoded protein products, then used to affinity-purify the cross-reacting antibody from the total anti-*Synechococcus* R2 cell wall preparation. This affinity-purified antibody could then be used to identify the original protein product of *Synechococcus* R2 cell walls and investigate the phenotype of mutants - constructed by disrupting the gene sequence - defective in a known outer membrane protein.

The further study of the mechanism of bacterial protein export, requires a balance between an analysis of the physiological process and the genetic characteristics which underlie this mechanism. In cyanobacteria both of these require attention, since it is possible that cyanobacteria have diverse mechanisms of such a process within the group. The genetic approach developed during the latter part of this thesis offers a means of analysis and can benefit from the extensive work carried out with other microbial export systems. However, there are only a limited number of cyanobacteria in which gene transfer is possible and of these the range of genetic 'tricks' is limited, hence it could be argued that the expansion of these areas is one of the most important aspects of future cyanobacterial research.

References

Adams, D. G. & Carr, N. G. (1981). The developmental biology of heterocyst and akinete formation in cyanobacteria. Crit. Rev. Microbiol. 9, 45-100.

Allen, M. M. (1968). Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4, 1-4.

Ames, G. F-L. (1986). Bacterial periplasmic transport systems: structure, mechanism and evolution. Ann. Rev. Biochem. 55, 397-425.

Ames, G. F-L., Prody, C. & Kustu, S. (1984). Simple, rapid and quantitative release of periplasmic proteins by chloroform. J. Bacteriol. 160, 1181-1183.

Andro, T., Chambost, J-P., Kotoujansky, A., Cattaneo, J., Bertheau, Y., Barras, F., van Gijsegem, F. & Coleno, A. (1984). Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. J. Bacteriol. 160, 1199-1203.

Antarikanonda, P. (1984). Production of extracellular free amino acids by cyanobacterium *Anabaena siamensis* Antarikanonda. Current Microbiology 11, 191-196.

Balbas, P., Soberon, X., Merino E., Zurita, M., Lomeli, H., Valle, F., Flores, F. & Bolivar, F. (1986). Plasmid vector pBR322 and its special purpose derivatives - a review. Gene 50, 3-40.

Bankaitis, V. A., Ryan, J. P., Rasmussen, B. A. & Bassford Jr., P. J. (1985). The use of genetic techniques to analyse protein export in *E. coli*. Curr. Top. Memb. Transp. 24, 106-146.

Bayer, M. E. (1979). The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. In: Bacterial Outer Membranes: Biogenesis and Function. M. Inouye (ed.), pp. 167-202. John Wiley & Sons Inc., New York.

Benson, S. A. (1985). Sequence information required for bacterial protein export. Bioessays 2, 201-205.

Benz, R. & Bohme, H. (1985). Pore formation by an outer membrane protein of the cyanobacterium *Anabaena variabilis*. Biochim. et Biophys. Acta 812, 286-292.

Benz, R., Woitzik, D., Flammann, H. T. & Wackesser, J. (1987). Pore forming activity of the major outer membrane protein of *Rhodobacter capsulatus* in lipid bilayer membranes. Arch. Microbiol. 148. 226-230.

Berg, D. E. & Berg, C. M. (1983). The prokaryotic transposable element Tn5. Biotechnology 1, 417-435.

Bergey's Manual of Systematic Bacteriology (1988, in press). Volume III. Williams & Wilkins (eds.).

Beringer, J. E., Beynon, J. L. Buchanan-Wollaston, A. V. & Johnston, A. W. B. (1978). Transfer of the transposon Tn5 to Rhizobium. Nature 276, 633-634.

Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Research 7, 1513-1523.

de Boer, H. A. & Shephard, H. M. (1983). Strategies for optimizing foreign gene expression in *E. coli*. In: 'Genes, Structure & Expression', A. M. Kroon (ed), pp. 205-248. Wiley, N.Y.

Bonen, L. & Doolittle, W. F. (1976). Nature 261, 669-673. Partial sequences of 16S rRNA and the phylogeny of blue-green algae and chloroplasts. Nature 261. 669-673.

Boquet, P. L., Manoil, C. & Beckwith, J. (1987). Use of Tn*phoA* to detect genes for exported proteins in *Escherichia coli*: identification of the plasmid-encoded gene for a periplasmic acid phosphatase. J. Bacteriol. 169. 1663-1669.

Bottomley, P. J. & Van Baalen, C. (1978). Characteristics of heterotrophic growth in the blue-green algae *Nostoc* sp. strain MAC. J. Gen. Micro. 107.309-318.

Bowyer, J. W. & Skerman, V. B. D. (1968). Production of axenic cultures of soil-borne and endophytic blue-green algae. J. Gen. Micro. 54. 299-306.

Boyer, G. L., Gillham, A. H. & Trick, C. (1987). Iron chelation and uptake. In: 'The Cyanobacteria', P. Fay & C. Van Baalen (eds), pp. 415-436. Elsevier, Netherlands.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brand, J. J. & Sheng, J-S. (1987). Polypeptide rapid turnover and photosystem II recovery from calcium stress in *Anacystis nidulans*. In: 'Progress in Photosynthesis Research' Vol. IV. J. Biggins (ed.), pp. 789-792. Martinus Nijhoff, Netherlands.

Braun, J. & Schmitz, G. (1980). Excretion of a protease by *Serratia marcescens*. Arch. Microbiol. 124. 55-61.

Brickman, E. & Beckwith, J. (1975). Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ø80 transducing phages. J. Mol. Biol. 96, 307-316.

Broome-Smith, J. K. & Spratt, B. G. (1986). A vector for the construction of translational fusions to TEM β-lactamase, the analysis of protein export signals and membrane protein topology. Gene 49. 341-349.

Bryant, D. A. (1986). The Cyanobacterial Photosynthetic Apparatus: Comparisons to those of higher plants and photosynthetic bacteria. In: 'Photosynthetic Picoplankton. T. Platt & W. K. W. Li (eds.), pp.423-500. Canadian Bulletin of Fisheries and Aquatic Science 214: 583pp.

Bullerjahn, G. S. (1985). Genes and gene transfer in Cyanobacteria (Review of Workshop). Plant Molecular Biology Reporter 3, 24-32.

Bullerjahn, G. S. & Sherman, L. A. (1986). Identification of a carotenoid-binding protein in the cytoplasmic membrane from the heterotrophic cyanobacterium *Synechocystis* sp. strain PCC6714. J. Bacteriol. 167, 396-399.

Burger-Wiersma, T., Veenhuis, M., Korthals, H. J., Van de Wiel, C. C. M. & Mur, L. R. (1986). A new prokaryote containing chlorophylls a and b. Nature 320, 262-264.

Buzby, J. S., Porter, R. D. & Stevens Jr., E. (1985). Expression of the *Escherichia coli* lac 2 gene on a plasmid vector in a cyanobacterium. Science 230, 805-807.

Casadaban, M. J. & Cohen, S. N. (1980). Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. 133, 179-207.

Casadaban, M. J. & Chou, J. (1984). *In vivo* formation of gene fusions encoding hybrid β-galactosidase proteins in one step with a transposable Mu-lac transducing phage. Proc. Nat. Acad. Sci. USA 81, 535-539.

Castell, J. V., Cervera, M. & Marlo, R. (1979). A convenient micromethod for the assay of primary amines and proteins with fluorescamine. A re-examination of the conditions of reaction. Anal Biochem. 99, 379-391.

Chart, H., Buck, M., Stevenson, P. & Griffiths, E. (1986). Iron regulated outer membrane proteins of *Escherichia coli*: variations in expression due to the chelator used to restrict the availability of iron. J. Gen. Micro. 132, 1373-1378.

Chauvat, F., Astier, C., Vedel, F. & Joset-Espardellier, F. (1983). Transformation in the cyanobacterium *Synechococcus* R2: improvement of efficiency; role of the pUH24 plasmid. Mol. Gen. Genet. 191, 39-45.

Chrost, R. J. & Brzeska, D. (1978). Extracellular release of organic products and growth of bacteria in *Anabaena cylindrica* (blue-green alga) culture. Acta Microbiol. Polon. 27, 287-295.

Chung, M. C-M. (1985). A specific iron stain for iron-binding in polyacrylamide gels: application to transferrin and lactoferrin. Anal. Biochem. 148, 498-502.

Clarke, S. E., Stuart, J. & Sanders-Loehr, J. (1987). Induction of siderophore activity in *Anabaena* spp. and its moderation of copper toxicity. App. Env. Micro. 53, 917-922.

Clement, J-M., Perrin, D. & Hedgpeth, J. (1982). Analysis of λ receptors and β-lactamase synthesis and export using cloned genes on a mini cell system. Mol. Gen. Genet. 185, 302-310.

Clewell, D. B. & Helinski, D. R. (1970). Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry 9, 4428-4440.

Codd, G. A. (1984). The Toxins of Cyanobacteria. Microbiol Sci. 1, 48-52.

Craig, R. & Reichelt, B. Y. (1986). Genetic engineering in algal biotechnology. Trends Biotechnol. 4, 280-285.

Daniell, H., Sarosini, G. & McFadden, B. A. (1986). Transformation of the cyanobacterium *Anacystis nidulans* 6301 with the *Escherichia coli* plasmid pBR322. Proc. Nat. Acad. Sci. USA 83, 2546-2550.

David, K. A. V. & Thomas, J. (1979). Extracellular polypeptides of *Anabaena* L-31: evidence for their role in regulation of heterocyst formation. J. Biosci. 1, 447-455.

Delaney, S. F. & Haichelt, B. Y. (1982). IV International Symposium on Photosynethic Prokaryotes D5. Integration of the R plasmid, R.68.45 into the genome of *Synechococcus* PCC6301.

Doolittle, W. F. (1979). J. G. Morris & Rose, A. H. (eds). The cyanobacterial genome, its expression, and the control of that expression. Advances in Microbial Physiology 20, 1-102.

Doolittle, W. G. (1982). Molecular evolution. In: 'The Biology of Cyanobacteria'. N. G. Carr & B. A. Whitton (eds.), pp. 307-331. Blackwell, Oxford.

Doonan, B. R. & Jensen, T. E. (1980). Ultrastructural localization of alkaline phosphatase in the cyanobacteria *Coccochloris peniocystis* and *Anabaena cylindrica*. Protoplasma 102, 189-197.

Drews, G. & Weckesser, J. (1982). Function, structure and composition of cell walls and external layers. In: 'The Biology of Cyanobacteria'. N. G. Carr & B. A. Whitton (eds.), pp. 333-357. Blackwell, Oxford.

Dzelzkalns, V. A., Owens, G. C. & Bogorad, L. (1984). Chloroplast promoter driven expression of the chloramphenicol acetyltransferase gene in a cyanobacterium. Nucleic Acids Research 23, 8917-8925.

Dzelzkalns, V. A. & Bogorad, L. (1986). Stable transformation of the cyanobacterium *Synechocystis* sp. PCC6803 induced by UV irradiation. J. Bacteriol. 165, 964-971.

Edelman, A., Bowler, L., Broome-Smith, J. K. & Spratt, B. G. (1987). Use of a β-lactamase fusion vector to investigate the organization of penicillin-binding protein 1B in the cytoplasmic membrane of *Escherichia coli*. Molecular Microbiology 1, 101-106.

Elhai, J. & Wolk, C. P. (1987). Proceedings of the Molecular Biology of Cyanobacteria Workshop, St. Louis, USA.

Elanskaya, I. V., Morzunova, I. B. & Shestakov, S. V. (1985). Expression of Gram-positive bacteria genes in cyanobacterium *Anacystis nidulans* R2. In: 'Abst. V Inter. Symp. Photosyn. Prok. Grindelwald, Switzerland. p.372.

Estep, M., Armstrong, J. E. & Van Baalen, C. (1975). Evidence for the occurrence of specific iron (III)-binding compounds in near-shore marine ecosystems. Appl. Microbiol. 30, 186-188.

Euteneuer, B. & Loos, M. (1985). Comparison of the exoproducts of Gram-negative bacteria by SDS-PAGE. Zbl. Bakt. Hyg. A. 259. 11-19.

Evans, E. H., Carr, N. G., Rush, J. D. & Johnson, C. E. (1977). Identification of a non-magnetic iron centre and an iron-storage or transport material in blue-green algal membranes by Mossbauer spectroscopy. Biochem. J. 166. 547-551.

Fattom, A. & Shilo, M. (1984). *Phormidium* J-1 bioflocculant: production and activity. Arch. Microbiol. 139. 421-426.

Feinberg, A. P. & Vogelstein, B. (1984). Addendum: a technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137. 266-267.

Flores, E. & Wolk, C. P. (1986). Production, by filamentous, nitrogen-fixing cyanobacteria of a bacteriocin and other antibiotics that kill related strains. Arch. Microbiol. 145. 215-219.

Fogg, G. E. (1952). The production of extracellular nitrogenous substances by a blue-green alga. Proc. R. Soc. B. 139. 372-397.

Fogg, G. E. (1971). Extracellular products of algae in freshwater. Arch. Hydrobiol. Beih. Ergebn. Limnol. 5, 1-25.

Fogg, G. E., Stewart, W. D. P., Fay, P. & Walsby, A. E. (1973). The Blue-Green algae. Academic Press.

Friedberg, D. & Saijffers, J. (1986). Controlled gene expression utilizing lambda phage regulatory signals in a cyanobacterium host. Mol. Gen. Genet. 203, 505-510.

Gallacher, M. L. & Burke Jnr., W. F. (1985a). Sequence-specific endonuclease from the transformable cyanobacterium *Anacystis nidulans* R2. FEMS Microbiol. Lett. 26, 317-321.

Gallacher, M. L. & Burke Jnr., W. F. (1985b). Expression by *Anacystis nidulans* R2 of an antibiotic resistance gene originating from a Gram-positive organism. Abstract H155, ASM 85th Annual Meeting.

Gardel, C., Benson, S., Hunt, J., Michaelis, S. & Beckwith, J. (1987). SecD, a new gene involved in protein export in *Escherichia coli*. J. Bacteriol. 169, 1286-1290.

Gendel, S., Straus, N., Pulleybank, D. & Williams, J. (1983). A novel shuttle cloning vector for the cyanobacterium *Anacystis nidulans*. FEMS Microbiol. Lett. 19, 291-294.

Gendel, S. M. (1987). Instability of Tn5 inserts in cyanobacterial cloning vectors. J. Bacteriol. 169, 4426-4430.

Gleason, F. K. & Paulson, J. L. (1984). Site of action of the natural algicide, cyanobacterin, in the blue-green alga, *Synechococcus* sp. Arch. Microbiol. 138, 273-277.

Golden, S. S. & Sherman, L. A. (1984). Optimal conditions for genetic transformation of the cyanobacterium *Anacystis nidulans* R2. J. Bacteriol. 158, 36-42.

Gray, G. L., Baldridge, J. S., McKeown, K. S., Heyneker, H. L. & Chang, C. N. (1985). Periplasmic production of correctly processed human growth hormone in *Escherichia coli*: natural and bacterial signal sequences are interchangeable. Gene 39, 247-254.

Grigorieva, G. A. & Shestakov, S. V. (1976). Application of the genetic transformation method for taxonomic analysis of unicellular blue-green algae. In: Proceedings of the 2nd International Symposium on Photosynthetic Prokaryotes, pp. 220-222, Dundee, Scotland. G. A. Codd & W. D. P. Stewart (eds.).

Hames, B. D. & Rickwood, D. (eds.). (1983). Gel Electrophoresis of Proteins - A Practical Approach. IRL Press, Oxford.

Hancock, R. E. W. (1987). Role of porins in outer membrane permeability. J. Bacteriol. 169. 929-933.

Hardie, L. P., Balkwill, D. L. & Stevens Jnr., S. E. (1983). Effects of iron starvation on the ultrastructure of the cyanobacterium *Agmenellum quadruplicatum*. Appl. Env. Microbiol. 42, 1007-1017.

Healey, F. P. (1973). Characteristics of phosphorous daficiency in *Anabaena*. J. Phycol. 9, 383-394.

Hellebust, J. A. (1974). Extracellular products. In. Algal physiology and biochemistry. W. D. P. Stewart (ed.), pp. 838-863. Blackwells Scientific Publications, Oxford.

Hengge, R. & Boos, W. (1984). Maltose and lactose transport in *Escherichia coli*. Examples of two different types of concentrative transport systems. Biochim. et Biophys. Acta 733. 443-478.

Herbst, V. & Overbeck, J. (1978). Metabolic coupling between the alga *Oscillatoria redekei* and accompanying bacteria. Naturwissenschaften 65. 598-599.

Herdman, M., Janvier, M., Rippka, R. & Stanier, R. Y. (1979). Genome size of cyanobacteria. J. Gen. Micro. 111. 73-85.

Herdman, M. (1982). Evolution and Genetic Properties of the Genome. In: 'The Biology of Cyanobacteria. N. G. Carr & B. A. Whitton (eds.), pp. 263-305. Blackwell.

Herrero, E., Jackson, M., Bassford, P. J., Sinden, D. & Holland, I. B. (1982). Insertion of a MalE β-galactosidase fusion protein into the envelope of *Escherichia coli* disrupts biogenesis of outer membrane proteins and processing of inner membrane proteins. J. Bacteriol. 152, 133-139.

Hinton, J. C. D. (1986). Development and application of molecular genetic techniques in *Erwinia carotovora* subsp. *carotovora*. Ph.D. Thesis, University of Warwick.

Hinton, J. C. D. & Salmond, G. P. C. (1987). Use of Tn*phoA* to enrich for extracellular enzyme mutants of *Erwinia carotovora* subspecies *carotovora*. Molecular Microbiology 1, 381-386.

Hobot, J. A., Carleman, E., Villiger, W. & Kellenberger, E. (1984). Periplasmic gel: new concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. J. Bacteriol. 160, 143-152.

Hoffman, C. S. & Wright, A. (1985). Fusions of secreted proteins to alkaline phosphatase: An approach to studying protein secretion. Proc. Nat. Acad. Sci. USA 82, 5107-5111.

Hofstra, H. & Dankert, J. (1979). Antigenic cross-reactivity of major outer membrane proteins in Enterobacteriaceae species. J. Gen. Micro. 111, 293- 302.

Holland, I. B, Mackman, N. & Nicaud, J-M. (1986). Secretion of proteins from bacteria. Biotechnology 4, 427-431.

van den Hondel, C. A. M. J. J., Keegstra, W., Borrias, W. E. & van Arkel, G. A. (1979). Homology of plasmids in strains of unicellular cyanobacteria. Plasmid 2, 323-333.

van den Hondel, C. A. M. J. J., Verbeek, S., van der Ende, A., Weisbeek, P. J., Borrias, W. E. & van Arkel, G. A. (1980). Introduction of transposon Tn*901 into a plasmid of *Anacystis nidulans*: preparation for cloning in cyanobacteria. Proc. Nat. Acad. Sci. USA 77, 1570-1574.

Hutber, G. N., Hutson, K. G. & Rogers, L. J. (1977). Effect of iron deficiency on levels of two ferredoxins and flavodoxin in a cyanobacterium. FEMS Microbiol. Lett. 1, 193-196.

Ito, K., Bassford Jnr., P. J. & Beckwith, J. R. (1981). Protein localization in *E. coli*: is there a common step in the secretion of periplasmic and outer membrane proteins? Cell 24, 707-717.

Itzhaki, R. F. & Gill, D. M. (1964). A micro-biuret method for estimating protein. Anal. Biochem. 9, 401-410.

Jones, G. E., Murray, L. & Carr, N. G. (1978). Trace element composition of five cyanobacteria. In: 'Environmental Biogeochemistry and Geomicrobiology'. Volume 3. Methods, metals and assessment. W. E. Krumbein (ed.). Ann. Arbor Science, Mich., USA.

Jurgens, U. J. & Weckesser, J. (1985). The fine structure and chemical composition of the cell wall and sheath layers of cyanobacteria. Ann. Inst. Pasteur/Microbiol. 136A, 41-44.

Kirby, N. W., Musgrave, S. C., Rowell, P., Shestakov, S. V. & Stewart, W. D. P. (1986). Photoproduction of ammonium by immobilized mutant strains of *Anabaena variabilis*. Appl. Microbiol. Biotechnol. 24, 42-46.

Klausner, A. (1986). Algaculture: food for thought. Biotechnology 4, 947-951.

Klebba, P. E., McIntosh, M. A. & Neilands, J. B. (1982). Kinetics of biosynthesis of iron-regulated membrane proteins in *Escherichia coli*. J. Bacteriol. 149, 880-888.

Kleckner, N. (1981). Transposable elements in prokaryotes. Ann. Rev. Genetics 15, 341-404.

Kolowsky, K. S., Williams, J. G. K. & Szalay, A. A. (1984). Length of foreign DNA in chimeric plasmids determines the efficiency of its integration into the chromosome of the cyanobacterium *Synechococcus* R2. Gene 27, 289-299.

Kroos, L. & Kaiser, D. (1984). Construction of Tn lac, a transposon that fuses lacZ expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc. Nat. Acad. Sci. USA 81, 5816-5820.

Kuhlemeier, C. J., Borrias, W. E., van den Hondel, C. A. M. J. J. & van Arkel, G. A. (1981). Vectors for cloning in cyanobacteria: construction and characterization of two recombinant plasmids capable of transformation to *Escherichia coli* K12 and *Anacystis nidulans* R2. Mol. Gen. Genet. 184, 249-254.

Kuhlemeier, C. J., Thomas, A. A. M., van der Ende, A., van Leen, R. W., Borrias, W. E., van den Hondel, C. A. M. J. J. & van Arkel, G. A. (1983). A host-vector system for gene cloning in the cyanobacterium *Anacystis nidulans* R2. Plasmid 10, 156-163.

Kushner, D. J. & Breuil, C. (1977). Penicillinase (β-lactamase) formation by blue-green algae. Arch. Microbiol. 112, 219-223.

Lammers, P. J. & Sanders-Loehr, J. (1982). Active transport of ferric schizokinen in *Anabaena* sp. J. Bacteriol. 151, 288-294.

Lange, W. (1974). Chelating agents and blue-green algae. Can. J. Microbiol. 20, 1311-1321.

Lazzaroni, J. C. & Portalier, R. C. (1985). Regulation of the tkyB gene expression in *Escherichia coli* K12 strains carrying a tkyB-lacZ gene fusion. Mol. Gen. Genet. 201, 323-328.

Legerski, R. J. & Robberson, D. L. (1985). Analysis and optimization of recombinant DNA joining reactions. J. Mol. Biol. 181. 297-312.

Lewin, R. A. (1976). Prochlorophyta as a proposed new division of algae. Nature 261. 697-698.

Lightfoot, D. A. (1985). Isolation and characterization of nitrogen assimilation genes in a cyanobacterium. Ph.D. Thesis, University of Leeds.

Lightfoot, D. A., Walters, D. E. & Wootton, J. C. (in press). Transformation of the cyanobacterium *Synechococcus* PCC6301 using cloned DNA. J. Gen. Micro.

Lind, L. K., Kalla, S. R., Lonneborg, A., Oquist, G. & Gustafsson, P. (1985). Cloning of the β-phycocyanin gene from *Anacystis nidulans*. FEBS Letts. 188. 27-32.

Lodge, J. M. T., Williams, P. & Brown, M. R. W. (1986). Influence of growth rate and iron limitation on the expression of outer membrane proteins and enterobactin by *Klebsiella pneumoniae* grown in continuous culture. J. Bacteriol. 165. 353-356.

de Lorenzo, V., Wee, S., Herrero, M. & Neilands, J. B. (1987). Operator sequences of the aerobactin operon of plasmid Col V-K30 binding the ferric uptake regulation (fur) repressor. J. Bacteriol. 169. 2624-2630.

Lory, S., Tai, P. C. & Davis, B. D. (1983). Mechanism of protein excretion by Gram-negative bacteria: *Pseudomonas aeruginosa* Exotoxin A. J. Bacteriol. 156. 2624-2630.

Manoil, C. & Beckwith, J. (1985). TnphoA: A transposon probe for protein export signals. Proc. Nat. Acad. Sci. USA 82. 8129-8133.

Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982). Molecular Cloning - A Laboratory Manual. Cold Spring Harbor Laboratory.

Masamoto, K., Reithman, H. C. & Sherman, L. A. (1987). Isolation and characterization of a carotenoid associated thylakoid protein from the cyanobacterium *Anacystis nidulans* R2. Plant Phys. 84, 633-639.

Mason, C. F., Edwards, K. R., Carlson, R. E., Pignatello, J., Gleason, F. K. & Wood, J. M. (1982). Isolation of chlorine-containing antibiotic from the freshwater cyanobacterium *Scytonema hofmanni*. Science 215, 400-402.

Matsuyama, S-I., Inokuchi, K. & Mizushima, S. (1984). Promoter exchange between ompF and ompC, genes for osmoregulated major outer membrane proteins of *Escherichia coli* K-12. J. Bacteriol. 158, 1041-1047.

McFarlane, G. J. B., Machray, G. C. & Stewart, W. D. P. (1987). A simplified method for conjugal gene transfer into the filamentous cyanobacterium *Anabaena* sp. ATCC 27893. J. Microbiol. Meth. 6, 301-305.

McKnight, D. M. & Morel, F. M. M. (1979). Release of weak and strong copper-complexing agents by algae. Limnol. Oceanogr. 24, 823-837.

Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E. & Ausubel, F. M. (1982). Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by Tn5 mutagenesis. J. Bacteriol. 149, 114-122.

Meeks, J. C., Enderlin, C. S., Joseph, C. M., Chapman, J. S. & Lollar, M. W. (1985). Fixation of [^{13}N] N_2 and transfer of fixed nitrogen in the *Anthoceros Nostoc* symbiotic association. Planta 164, 406-414.

Michaelis, S. & Beckwith, J. (1982). Mechanism of incorporation of cell envelope proteins in *Escherichia coli*. Ann. Rev. Microbiol. 36, 435-465.

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Moore, S. & Stein, W. H. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. 176. 367.

Nagahari, K., Kanaya, S., Munakata, K., Aoyagi, Y. & Mizushima, S. (1985). Secretion into the culture medium of a foreign gene product from *Escherichia coli*: use of the ompF gene for secretion of human β-endorphin. EMBO J. 4, 3589-3592.

Nahlik, M. S., Fleming, T. P. & McIntosh, M. A. (1987). Cluster of genes controlling synthesis and activation of 2,3-dihydroxybenzoic acid in production of enterobactin in *E. coli*. J. Bacteriol. 169, 4163-4170.

Nakamura, K. & Mizushima, S. (1976). Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from *Escherichia coli* K-12. J. Biochem. 80, 1411-1422.

Nara, F., Matsuyama, S., Mizuno, T. & Mizushima, S. (1986). Molecular analysis of mutant ompR genes exhibiting different phenotypes as to osmoregulation of the ompF and ompC genes of *Escherichia coli*. Mol. Gen. Genetc. 202, 194-199.

Neilands, J. B. (1984). Siderophores of bacteria and fungi. Microbiol. Sci. 1, 9-14.

Neilands, J. B. & Nakamura, K. (1985). Regulation of iron assimilation in microorganisms. Nutrition Reviews 43, 193-197.

Neu, H. C. & Heppel, L. A. (1965). The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of sphaeroplasts. J. Biol. Chem. 240. 3685-3692.

Nicaud, J-M., Breton, A., Younes, G. & Guespin-Michel, J. (1984). Mutants of *Myxococcus xanthus* impaired in protein secretion: an approach to study of a secretory mechanism. Appl. Microbiol. Biotechnol. 20. 344-350.

Nicaud, J-M., Mackman, N. & Holland, I. B. (1986). Minireview. Current status of secretion of foreign proteins by microorganisms. J. Biotech. 3, 255-270.

Omata, T. & Murata, N. (1984). Isolation and characterization of three types of membranes from the cyanobacterium (blue-green alga) *Synechocystis* PCC6714. Arch. Microbiol. 139, 113-116.

Omata, T. & Ogawa, T. (1986). Biosynthesis of a 42-kD polypeptide in the cytoplasmic membrane of the cyanobacterium *Anacytis nidulans* strain R2 during adaptation to low CO_2 concentration. Plant Physiol. 80, 525-530.

Ozenberger, B. A., Nahlik, M. S. & McIntosh, M. A. (1987). Genetic organization of multiple *fep* genes encoding ferric enterobactin transport functions in *Escherichia coli*. J. Bacteriol. 169. 3638-3646.

Padan, E. & Cohen, Y. (1982). Anoxygenic photosynthesis. In: 'The Biology of Cyanobacteria'. N. G. Carr & B. A. Whitton (eds.), pp. 215-235. Blackwell, Oxford.

Paerl, H. W. (1978). Role of heterotrophic bacteria in promoting N_2 fixation by *Anabaena* in aquatic habitats. Micro. Ecol. 4, 215-231.

Pakrasi, H. B., Goldenberg, A. & Sherman, L. A. (1985). Membrane development in the cyanobacterium, *Anacystis nidulans*, during recovery from iron starvation. Plant Physiol. 79, 290-295.

Palva, E. T. & Silhavy, T. J. (1984). LacZ fusions to genes that specify exported proteins: A general technique. Mol. Gen. Genet. 196. 388-394.

Poole, K. & Hancock, R. E. W. (1986). Phosphate-starvation induced outer membrane proteins of members of the families *Enterobacteriaceae* and *Pseudomonaceae*: Demonstration of immunological cross-reactivity with an antiserum specific for porin protein P of *Pseudomonas aeruginosa*. J. Bacteriol. 165. 987-993.

Porter, R. D. (1986). Transformation in cyanobacteria. CRC Crit. Rev. Microbiol. 13, 111-132.

Pribnow, D. (1975). Bacteriophage T7 early promoters nucleotide sequences of two RNA polymerase binding sites. J. Mol. Biol. 99, 419-443.

Pugsley, A. P. (1984). The ins and outs of colicins, Part I. Production and translocation across membranes. Microbiol. Sci. 1, 168-175.

Pugsley, A. P. & Schwartz, M. (1985). Export and secretion of proteins by bacteria. FEMS Microbiol. Revs. 32, 3-38.

Pugsley, A. P. & Cole, S. T. (1986). β-galactosidase and alkaline phosphatase do not become extracellular when fused to colicin. J. Gen. Micro. 132, 2297-2307.

Reaston, J., van den Hondel, C. A. M. J. J., van der Ende, A., van Arkel, G. A., Stewart, W. D. P. & Herdman, M. (1980). Comparison of plasmids from the cyanobacterium *Nostoc* PCC7524 with two mutant strains unable to form heterocysts. FEMS Microbiol. Lett. 9, 185-188.

Reddy, K. J., Vann, C. & Sherman, L. (1987). Cloning and characterization of a gene encoding an iron-regulated membrane protein in *Anacystis nidulans* R2. In: 'Progress in Photosynthesis Research' Vol. IV. J. Biggins (ed.), pp.777-780.

Reiss, B., Sprengel, R., Will, H. & Schaller, H. (1984). A new sensitive method for qualitative and quantitative assay of neomycin phosphotransferase in crude cell extracts. Gene 30, 211-218.

Resch, C. M. & Gibson, J. (1983). Isolation of the carotenoid-containing cell wall of three unicellular cyanobacteria. J. Bacteriol. 155, 345-350.

Reverchon, S., van Gijsegem, F., Rouve, M., Kotoujnsky, A. & Robert-Baudouy, J. (1986). Organization of a pectate lyase gene family in *Erwinia chrysanthemi*. Gene 49, 215-224.

Rippka, R., Deruelles, J., Waterbury, J. B., Herdman, M. & Stanier, R. Y. (1979). Genetic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol. 111, 1-61.

Rosen, B. P. (ed.) (1978). Bacterial Transport. Marcel Dekker, New York.

Salmond, G. P. C., Hinton, J. C. D., Gill, D. R. & Parombalon, M. C. M. (1986). Transposon mutagenesis of *Erwinia* using phage λ vectors. Mol. Gen. Genet. 203, 524-528.

Schmatterer, G., Wolk, C. P. & Elhai, J. (1986). Expression of luciferases from *Vibrio harveyi* and *Vibrio fischeri* in filamentous cyanobacteria. J. Bacteriol. 167, 411-414.

Schmid, M. B. & Roth, J. R. (1987). Gene location affects expression level in *Salmonella typhimurium*. J. Bacteriol. 169, 2872-2875.

Schnaitman, C. A. (1981). Cell fractionation. In: 'Manual of Methods for General Bacteriology'. P. Gerhaerdt (ed.), pp.52-61. American Society of Microbiology, Washington DC.

Schneider, K. & Beck, C. F. (1986). Promoter probe vectors for the analysis of divergently arranged promoters. Gene 42, 37-48.

Schwyn, B. & Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 37-48.

Shestakov, S. V,. & Khyen, N. T. (1970). Evidence for genetic transformation in the blue-green alga *Anacystis nidulans*. Mol. Gen. Genet. 107, 372-375.

Shestakov, S. V. & Reaston, J. (in press). Gene transfer and host-vector systems of cyanobacteria. Oxford Surveys of Plant Mol. Cell Biol. 4.

Siefermann-Harms, D. (1987). The light-harvesting and protective functions of carotenoids in photosynthetic membranes. Physiol. Plantarum 69, 561-568.

Silhavy, T. J. & Beckwith, J. R. (1985). Uses of lac fusions for the study of biological problems. Microbiol. Revs. 49, 348-418.

Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. Biotechnology 1, 784-791.

Singh, H. N. & Sonie, K. C. (1977). Isolation and characterization of chlorate-resistant mutants of the blue-green alga *Nostoc muscorum*. Mutation Res. 43, 205-212.

Smith, H., Bron, S., van Ea, J. & Venema, G. (1987). Construction and use of signal sequence selection vectors in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 169, 3321-3328.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503.

Stanier, R. Y. & Cohen-Bazire, G. (1977). Phototrophic prokaryotes: the cyanobacteria. Ann. Rev. Microbiol. 31, 225-274.

Starr, M. P., Chatterjee, A. K., Starr, P. B. & Buchanan, G. E. (1977). Enzymatic degradation of polygalacturonic acid by *Yersinia* and *Klebsiella* spp. in relation to clinical laboratory procedures. J. Clin. Microbiol. 6, 379-386.

Stevens, S. E. & Porter, R. D. (1980). Transformation in *Agmenellum quadruplicatum*. Proc. Nat. Acad. Sci. USA 77, 6052-6056.

Stewart, W. D. P. (1964). Nitrogen fixation by *Myxophyceae* from marine environments. J. Gen. Micro. 36, 415-422.

Subramanian, G. & Shanmugasundaram, S. (1986). Uninduced ammonia release by the nitrogen-fixing cyanobacterium *Anabaena*. FEMS Microbiol. Lett. 37, 151-154.

Tandeau de Marsac, N. (1977). Occurrence and nature of chromatic adaptation in cyanobacteria. J. Bacteriol. 130. 82-91.

Tandeau de Marsac, N., Borrias, W. E., Kuhlemeier, C. J., Castets, A. M., van Arkel, G. A. and van den Hondel, C. A. M. J. J. (1982). A new approach for molecular cloning in cyanobacteria: cloning of an *Anacystis nidulans met* gene using a Tn901-induced mutant. Gene 20. 111-119.

Tandeau de Marsac, N. & Houmand, J. (1987). Advances in Cyanobacterial Molecular Genetics. In: 'The Cyanobacteria'. P. Fay & C. Van Baalen (eds.), pp. 251-302. Elsevier, Netherlands.

Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Nat. Acad. Sci. USA 84, 2833-2837.

Tomioka, N. & Sugiura, M. (1983). The complete nucleotide sequence of the 16S ribosomal RNA gene from a blue-green alga, *Anacystis nidulans*. Mol. Gen. Genet. 191. 46-50.

Vasantha, N. & Thompson, L. D. (1986). Secretion of a heterologous protein from *Bacillus subtilis* with the aid of protease signal sequences. J. Bacteriol. 165. 837-842.

Villarejo, M. & Case, C. C. (1984). *envZ* mediates transcriptional control by local anaesthetics but is not required for osmoregulation in *Escherichia coli*. J. Bacteriol. 159. 883-887.

Walsby, A. E. (1974). The extracellular products of *Anabaena cylindrica* lemm. I. Isolation of a macromolecular pigment-peptide complex and other components. Br. Phycol. J. 9, 371-381.

Walsby, A. E. (1987). Mechanisms of buoyancy regulation by planktonic cyanobacteria with gas vesicles. In: 'The Cyanobacteria'. P. Fay & C. Van Baalen (eds.), pp. 377-392. Elsevier, Netherlands.

Wang, P-Z., Projan, S. J., Leason, K. R. & Novak, R. P. (1987). Translational fusion with a secretory enzyme as an indicator. J. Bacteriol. 169, 3082-3087.

Whitton, B. A. (1965). Extracellular products of blue-green algae. J. Gen. Micro. 40, 1-11.

Wiedmann, M., Kurzchalia, T., Hartmann, E. & Rapoport, T. A. (1987). A signal sequence receptor in the endoplasmic reticulum membrane. Nature 328, 830-833.

Williams, J. G. K. & Szalay, A. A. (1983). Stable integration of foreign DNA into the chromosome of the cyanobacterium *Synechococcus* R2. Gene 24, 37-51.

Wolk, C. P. (1980). Heterocysts, ^{13}N, and N_2-fixing plants. In: 'Nitrogen fixation', Vol. II. W. E. Newton, W. H. Orme-Johnson (eds.), pp. 279-292. University Park Press, Baltimore, M.D.

Wolk, C. P. & Kraus, J. (1982). Two approaches to obtaining low, extracellular deoxyribonuclease activity in cultures of heterocyst forming cyanobacteria. Arch. Microbiol. 131. 302-307.

Wolk, C. P., Vonshak, A., Kehoe, P., & Elhai, J. (1984). Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. Proc. Nat. Acad. Sci. USA 81, 1561-1565.

Wray, W., Boulikas, T., Wray, V. P. & Hancock, R. (1981). Silver staining of proteins in polyacrylamide gels. Anal. Biochem. 118. 197-203.

Yamanaka, G. & Glazer, A. N. (1981). Dynamic aspects of phycobilisome structure: modulation of phycocyanin content of *Synechococcus* phycobilisomes. Arch. Microbiol. 130. 23-30.

Young, R. A. & Davis, R. W. (1983). Efficient isolation of genes by using antibody probes. Proc. Nat. Acad. Sci. USA 80. 1194-1198.

Youngman, P., Zuber, P., Perkins, T. B., Sandman, K., Igo, M. & Losick, R. (1985). New ways to study developmental genes in spore-forming bacteria. Science 228. 285-291.

Zink, R. T., Kemble, R. J. & Chatterjee, A. K. (1984). Transposon Tn5 mutagenesis in *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica*. J. Bacteriol. 157. 809-814.

de Bruijn, F. J. & Lupski, J. R. (1984). The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids - a review.

Cohen-Bazire, G. & Bryant, D. A. (1982). Phycobilisomes: composition and structure. In: 'The Biology of Cyanobacteria'. N. G. Carr & B. A. Whitton (eds.), pp.143-190. Blackwell.

Simpson, F. B. & Neilands, J. B. (1976). Siderochromes in Cyanophyceae: isolation and characterization of schizokinen from *Anabaena* sp. J. Phycol. 12. 44-48.