THE MOLECULAR GENETICS OF PIGMENT BIOSYNTHESIS IN *Rhodobacter sphaeroides*

SHIRLEY ANN COOMBER

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Imperial College
Department of Pure and Applied Biology
London

ABSTRACT

Rhodobacter sphaeroides is a purple non-sulphur photosynthetic bacterium which is capable of chemoheterotrophic growth in the dark and anaerobic photosynthetic growth in the light. The ability of *Rb. sphaeroides* to grow chemoheterotrophically allows the study of lesions in genes encoding components of the photosynthetic apparatus, which in many other photosynthetic organisms would be fatal. Accordingly, *Rb. sphaeroides* has been widely used as a model organism to study various aspects of photosynthesis.

In this work a number of clones have been isolated from a bank of *Rb. sphaeroides* genomic DNA in *Escherichia coli* which, on transfer, restored a series of non-photosynthetic mutants to wild type phenotype. These clones form the basis of a 45 kb photosynthesis gene cluster. This cluster carries the genes for the committed steps of bacteriochlorophyll and carotenoid biosynthesis, and for the reaction centre and B875 light harvesting polypeptides.

Furthermore, this cluster was subjected to intensive site directed Tn5 mutagenesis. Over 70 Tn5 insertions have been positioned on the 45 kb cluster and each mutant phenotype characterized. Overall, the postion and extent of six carotenoid and nine bacteriochlorophyll biosynthesis genes have been identified.

The N1 mutant of *Rb. sphaeroides* excretes a haem /bacteriochlorophyll intermediate identified as coproporphyrin III. A clone was isolated from a bank of *Rb. sphaeroides* genomic DNA in *E. coli* which, on transfer, restored N1 to wild type phenotype. Site directed Tn5 mutagenesis was used and the position of insertions which produced a N1-like phenotype were mapped. 1.8 kb was sequenced and an open reading frame of 750 bp was identified which showed typical *Rb. sphaeroides* codon usage. Northern analysis showed that the 1.3 kb mRNA predicted from sequence analysis was produced. The mRNA transcript was negatively regulated by oxygen. Nuclease S1 analysis of the 5' end of the mRNA identified a sequence which showed strong similarity to the oxygen regulated *puf*1 promoter. Biochemical analysis of the N1 mutant has shown the absence of detectable coproporphyrinogen III oxidase activity. It is suggested that this open reading frame encodes a gene for an anaerobic coproporphyrinogen III oxidase and has been named *hem*F.

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ABBREVIATIONS

A	Absorbance
ALA	5-aminolevulinic acid
ALA-D	ALA dehydratase
ALA-S	ALA synthase
ATP	adenosine-5'-triphosphate
bchl	bacteriochlorophyll
bp	base pair(s)
BSA	bovine serum albumin
crt	carotenoid
cDNA	complementary DNA
Coprogen	coproporphyrinogen III
cyt	cytochrome
dATP	2'-deoyx-adenosine-5'-triphosphate
dCTP	2'-deoyx-cytidine-5'-triphosphate
dGTP	2'-deoyx-guanosine-5'-triphosphate
dTTP	2'-deoyx-thymidine-5'-triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol

DVPChlide	Mg 2,4-divinyl pheoporphyrin a_5 monomethyl ester
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl) - N,N,N',N'-tetraacetic acid
EtOH	ethanol
kb	kilobase pair(s)
kD	kilodalton(s)
LB	Luria-Bertani
LH	light harvesting
Mg-PME	Mg-protoporphyrin monomethyl ester
Mr	relative molecular mass
MOPS	4-morpholinepropane-sulphonic acid
NADH	reduced ß-nicotinamide adenine dinucleotide
NADPH	reduced ß-nicotinamide adenine dinucleotide phosphate
Nm	neomycin
PAGE	polyacrylamide gel electrophoresis
PBG	porphobilinogen

PChlide	Mg 2-vinyl, 4-ethyl pheoporphyrin a_5 monomethyl ester
Proto	protoporphyrin IX
Protogen	protoporphyrinogen IX
RC	reaction centre
PS	photosynthesis
RNA	ribonucleic acid
rpm	revolutions per minute
tRNA	transfer ribonucleic acid
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
SAM	s-adenosyl methionine
SDS	sodium dodecyl sulphate
ssDNA	single stranded DNA
TBE	Tris-borate-EDTA
TE	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
Tn	transposon
Tris	2-amino-2(hydroxymethyl)-1,3 - propandiol
Urogen	uroporphyrinogen III
uv	ultra violet

LIST OF PUBLICATIONS.

1. Shirley A. Coomber, Mark K. Ashby, and C. Neil Hunter (1987) 'Cloning and oxygen regulated expression of genes coding for the bacteriochlorophyll biosynthetic pathway in *Rhodobacter sphaeroides*.' in Progress in Photosynthesis Research, 4, pp737 -740. (Biggins, J. ed.) Martinus Nijhoff, Dordrecht.

2. Mark K. Ashby, Shirley A. Coomber and C. Neil Hunter (1987) 'Cloning and oxygen regulated expression of genes coding for the reaction centre and light-harvesting polypeptides of *Rhodobacter sphaeroides*.' in Progress in Photosynthesis Research, 4, pp733-736. (Biggins, J. ed.) Martinus Nijhoff, Dordrecht.

3. Mark K. Ashby, Shirley A. Coomber and C. Neil Hunter (1987). 'Cloning, nucleotide sequence and transfer of genes for the B800-850 light harvesting complex of *Rhodobacter sphaeroides*.' FEBS Lett. **213**, 245-248.

4. C. Neil Hunter, Mark K. Ashby and Shirley A. Coomber (1987). 'Effect of oxygen on levels of mRNA coding for reaction-centre and light-harvesting polypeptides of *Rhodobacter sphaeroides*.' Biochem. J. **247**, 489-492.

5. C. Neil Hunter and Shirley A. Coomber (1988). 'Cloning and oxygen-regulated expression of the bacteriochlorophyll biosynthesis genes *bch*E, B, A and C of *Rhodobacter sphaeroides*.' J. Gen. Microbiol. **134**, 1491-1497.

6. Shirley A. Coomber and C. Neil Hunter (1988). 'Construction of a physical map of the 45 kb photosynthetic gene cluster of *Rhodobacter sphaeroides*.' Submitted to Arch. Microbiol.

7. Peter J. Nixon, Kleoniki Gournaris, Shirley A. Coomber, C. Neil Hunter, Tristan A. Dyer and James Barber (1988). 'Immunological evidence against the product of *psb*G being a component in photosystem two.' Submitted EMBO. J.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1. Photosynthesis

Photosynthesis occurs in a wide range of organisms, including bacteria, cyanobacteria, algae and plants. The simplest equation for photosynthesis is shown in equation [1] (van Niel, 1962).

$$2H_2X + CO_2 \xrightarrow{\text{light}} (CH_2O) + H_2O + 2X \quad [1]$$

where H_2X is the source of hydrogen ions and electrons needed to reduce carbon dioxide to carbohydrate $(CH_2O)$and other organic molecules, and X is the oxidation product. In plants, red and brown algae, and cyanobacteria H_2O is the reductant and molecular oxygen the by-product. Photosynthetic bacteria use a wide range of reductants including H_2S, H_2, $Na_2S_2O_3$ and a variety of simple organic compounds. The first four steps of photosynthesis in photosynthetic bacteria can be categorised as follows:-

a) Energy capture: light is absorbed by pigment molecules and the excitation energy channelled to the reaction centre.

b) Charge separation: light energy is used to drive charge separation across the membrane at a reaction centre.

c) Electron transport: the charge separation initiates a series of redox reactions which result in the production ATP and reducing equivalents in the form of NADH.

d) Carbon assimilation: ATP and NADH are used in the reductive carboxylic acid cycle to fix CO_2.

A number of pigment types form essential components of the photosynthetic apparatus of bacteria. The chlorophyll pigments called bacteriochlorophylls (bchl) form part of the light harvesting and reaction centre complexes which are responsible for trapping photons of visible and near infrared light and converting this excitation energy to photochemical energy at the reaction centre where bchl participates in charge separation. Carotenoids (crt),found in the reaction centre (RC) and both light harvesting complexes (LHI and LHII) and have a light harvesting function; in addition they are thought to protect against photooxidative damage caused by the oxygen radicals formed from bchl in its excited state. Haem forms an essential component of the cytochrome bc_1 (cytbc_1) and cytochrome c_2 (cytc_2) complexes

which are part of the electron transport chain. The aim of this work is to study genetic aspects of the synthesis of these pigment groups essential for photosynthesis.

Rhodobacter sphaeroides was chosen as a model organism in which to study pigment biosynthesis. Historically, it has been widely used to identify the sequence of reactions in the tetrapyrrole biosynthesis pathway. *Rb. sphaeroides*, along with other purple nonsulphur bacteria can grow chemoheterotrophically as well as photosynthetically. This ability can be exploited to identify genes involved in photosynthetic membrane assembly including those for reaction centre and light harvesting polypeptides and the committed steps of bchl and crt biosynthesis. This thesis contains an account of the cloning and characterization of genes encoding enzymes of the bchl and crt biosynthesis pathways. To achieve this a number of genetic techniques were used for the first time in *Rb. sphaeroides*. It should be noted that *Rhodopseudomonas capsulata* and *Rhodopseudomonas sphaeroides* have been renamed *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* respectively (Imhoff *et al.*, 1984).

1.2. Biochemistry and genetics of the haem and bacteriochlorophyll biosynthesis

Haem and bacteriochlorophyll comprise part of a large family of biosynthetically related molecules called tetrapyrroles. Figure 1.1 shows the relationship of each member of the tetrapyrrole family in the biosynthetic pathway. Although no single organism is capable of forming all the different tetrapyrrole end products, two or more end products are often formed simultaneously or at different stages of development within a single organism.

The biochemical study of chlorophyll and haem biosynthetic pathways was initiated in the 1940's by Granick, who isolated several mutant strains of *Chlorella* that were deficient in chlorophyll biosynthesis. Some of these mutant strains accumulated porphyrins, a process that was shown to be regulated by haem (Granick, 1950). During the same period Shemin and his co-workers were carrying out isotopic studies on the precursors of porphyrins (Shemin and Wittenberg, 1951). Also Neuberger and his group, working predominantly with the purple non-sulphur bacterium *Rhodobacter sphaeroides*, identified a number of initial steps in the tetrapyrrole biosynthesis pathway (Neuberger and Scott, 1953). The discovery of the enzymes of the tetrapyrrole pathway is shared by these three groups.

In the 1960's two groups, Lascelles and co-workers and Jones also used *Rb. sphaeroides* to deduce the sequence of biochemical events from the point of Mg^{2+} insertion in bchl biosynthesis. The series of chemical steps leading to chlorophyll and haem biosynthesis can be divided into a number of distinct processes. Historically, the first intermediate that was thought to be unique to the tetrapyrrole pathway was 5-

aminolevulinic acid (ALA). The sequence of intermediates from ALA to bacteriochlorophyll *a* is shown in figure 1.2. Also indicated are the branch points for chlorophyll *a* and haem biosynthesis. The steps of ALA to protoporphyrin IX are common to both chlorophyll and haem biosynthesis with the insertion of Mg^{2+} and Fe^{2+} determining the ultimate production of a chlorophyll or haem molecule respectively. Figure 1.3A shows the variations of side chains which produce the various bacteriochlorophyll and chlorophyll molecules found, which share a common tetrapyrrole ring structure (Gloe *et al.*, 1975). The most well known bacteriochlorophyll form in photosynthetic bacteria, Bchl*a*, is found in *Rhodospirillaceae*; Bchl*b* has so far only been found in *Rhodopseudomonas viridis*. Bchl*c*, *d* and *e* are found in green sulphur bacteria (*Chlorobiaceae*).

Photosynthetic bacteria are rich in soluble and membrane bound c type cytochromes and many also contain forms of cyt b (Bartsch, 1978). Cyt a is rare among the photosynthetic bacteria and so far has only been found in aerobically grown *Rb. sphaeroides* (Saunders and Jones, 1974). Figure 1.3B shows the structure of hemes found in photosynthetic bacteria.

In general the enzymes for haem and chlorophyll biosynthesis have proved difficult to isolate to homogeneity. Recently, however a number of advances have been achieved in the study of the early enzymes of haem biosynthesis; some of these steps have been implicated in human porphyrias where abnormal enzyme activities lead to accumulation of porphyrin intermediates. Each step from the formation of ALA will be discussed separately with reference to photosynthetic organisms where possible. Later, the steps from Mg^{2+} insertion committed to chlorophyll/bacteriochlorophyll biosynthesis will be covered in detail.

1.2.1. 5-aminolevulinic acid formation

There are two basic routes for 5-aminolevulinic acid (ALA) biosynthesis, the Shemin pathway and the C_5 pathway. Despite much searching only one organism, *Euglena gracilis* (Weinstein and Beale, 1983) appears to be able to make ALA by both pathways.

1.2.1.1. The Shemin pathway

In the classical Shemin pathway (Figure 1.4A) 2-oxoglutarate is converted with the loss of carbon as CO_2 to succinyl-CoA, which is then condensed with glycine to give ALA and CO_2 by the enzyme 5-aminolevulinic acid synthase (ALA-S) (Shemin, 1956). This pathway was found in some bacteria including *Rhodobacter* sp., fungi, yeast and animals. The enzyme is thought to have both cytosolic and membrane

bound forms in *Rb. sphaeroides* (Fanica-Gaignier and Clément-Metral, 1973). In eukaryotes it is formed on cytosolic ribosomes and then transported into the mitochondrial matrix (Keng *et al.*, 1986). In *Rb. sphaeroides* enzymic activity is inhibited by haem (Burnham and Lascelles, 1963), magnesium protoporphyrin (Yubisui and Yoneyama, 1972), and light (Oelze and Arnheim, 1983a; Oelze, 1986). Although it has been suggested that both the C_5 and Shemin pathways operate in *Rb. sphaeroides* (Chen *et al.*, 1981), it has been shown using C13 and C14 NMR techniques, that only the Shemin pathway is utilized (Porra, 1986; Oh-hama *et al.*, 1985). Two 5-aminolevulinate requiring mutants have been isolated in *Rb. sphaeroides*. Although these mutants H4 and H5, do not have the same phenotypes, Lascelles and Altshuler (1969) concluded that only one ALA-S enzyme was present. Recently, Wright *et al.*, (1987) isolated a ALA requiring mutant of *Rb. capsulatus* using random Tn5 mutagenesis, and have since used transposon rescue techniques to clone *hem*A (Biel *et al.*, 1988). This mutant still had 10% of the wild type ALA-S activity, and though this could be due to the production of a truncated protein with reduced activity, it would also be consistent with the presence of two ALA-S enzymes encoded by different genes.

In animals two ALA-S genes have been isolated and sequenced. In mouse, a full length cDNA clone that was found to complement a *hem*A mutant of *E. coli* has been sequenced and found to encode a polypeptide of Mr=64,600. (Schoenhaut and Curtis, 1986). In chicken both the cDNA and the full length genomic sequence for this gene have been obtained encoding a polypeptide of Mr=70,000 (found to be 68,000 on SDS-PAGE) (Maguire *et al.*, 1986). At the amino acid level these genes show extensive homology, particularly at the carboxyl-terminal end (Schoenhaut and Curtis, 1986). It has been concluded that in chicken, ALA-S is encoded by a unique gene (Elferink *et al.*, 1987). Two partial amino terminal sequences of ALA-S have also been determined in *Rhizobium meliloti* (Leong *et al.*, 1985) and yeast (Keng *et al.*,1986).

1.2.1.2. The C_5 pathway

The 5-carbon compounds glutamate, α-ketoglutarate and glutamine were found to be more efficiently incorporated into ALA than glycine and succinate in greening tissues of cucumber, barley and bean (Beale and Castelfranco, 1974) and maize (Meller *et al.*, 1975). The uniform degree of incorporation of all the carbons of glutamate or α-ketoglutarate into ALA suggested that a new C_5 pathway for ALA formation was being utilized. The C_5 pathway of ALA synthesis (Figure 1.4B) has been found in plants and numerous bacterial species (see Kannangara *et al.*, 1988 for review). The

enzyme complex involved has been purified from barley (Wang *et al.*,1981) and has since been characterized in a number of organisms (Kannangara *et al.*, 1988).

In the first step of this pathway, glutamate is esterified to $tRNA^{Glu}$ by glutamic acid-tRNA ligase, which also provides glutamyl-tRNA for protein synthesis (Schön *et al.*,1986; Bryant and Kannangara, 1987). Glutamyl-tRNA is then reduced in a NADPH-requiring reaction. The dehydrogenase that catalyses this reaction is strongly inhibited by low concentrations of hemin (Weinstein and Beale, 1985; Huang and Wang, 1986), which suggests that this step is the site of feedback control of porphyrin biosynthesis. The product of this second reaction is glutamate l-semialdehyde (Houen *et al.*, 1983; Kannangara and Schouboe, 1985). Finally, ALA is formed from glutamate l-semialdehyde by an isomerization that is catalysed by an aminotransferase (Kannangara and Gough, 1978). Gabaculin (3-amino 2,3-dihydrobenzoic acid), a potent inhibitor of Ɣ-aminobutyrate (GABA) aminotransferase, has also been found to be an inhibitor of tetrapyrrole biosynthesis in plants (Flint, 1984; Hill *et al.*, 1985; Kannangara and Schouboe, 1985). Recent results have confirmed that the enzyme glutamate-1-semialdehyde aminotransferase is the site of gabaculin inhibition (Hoober *et al.*,1988). None of the genes encoding the three enzymes known to be involved in the C_5 pathway have been isolated.

1.2.2. Formation of porphobilinogen (PBG)

PBG was first found to be a porphyrin precursor in avian red blood cell preparations (Falk *et al.*, 1953) and in *Chlorella* (Bogorad and Granick, 1953). 5-aminolevulinate dehydratase (ALA-D) has been found in a large number of bacterial, fungal, plant and animal species (Castelfranco and Beale, 1981). ALA-D catalyses the condensation of two molecules of ALA to form the monopyrrole porphobilinogen (PBG). The reaction mechanism was first studied in detail in *Rb. sphaeroides*. Firstly, one molecule of ALA forms a Schiff's base with a lysine group of the enzyme, then after condensation with the second ALA molecule, PBG is released (Nandi and Shemin, 1968). ALA -dehydratase has been purified, in some cases to homogeniety, in a large number of species, and exhibits differing metal cofactor requirements. ALA-D from *Rb. sphaeroides* has been found to require K^+ (Burnham and Lascelles, 1963), whereas fungal and animal ALA-D require Zn^{2+} (Muthukrishan *et al.*, 1972). All plant derived enzymes are activated by Mg^{2+} or Mn^{2+} (Schneider, 1970).

In this instance the molecular genetics of the *hem*B gene encoding this step are more fully developed in eukaryotes. Although a clone complementing a *hem*B mutant of *E. coli* was recently reported (Li *et al.*, 1988), the availability of highly purified ALA-D enzyme has led to earlier isolation of the eukaryotic *hem*B gene. Antibodies raised against ALA-D from yeast and human sources have led to the

isolation and sequence of the full length cDNA clones of yeast *hem*B (Myers *et al.*, 1987) and the human *hem*B (Wetmur *et al.*, 1986) genes, encoding for polypeptides of Mr=37,837 and Mr=36,274 respectively. Along with the sequences of four peptides of bovine ALA-D (Lingner and Kleinschmidt, 1983) the two amino acid sequences from yeast and human show 52% identity (Myers *et al.*, 1987). A full length sequence of a cDNA clone of rat *hem*B has also been reported (Bishop *et al.*, 1986) which shows 88% homology, at amino acid level, with the human sequence.

1.2.3. The formation of Uroporphyrinogen III

The condensation of four molecules of PBG to form the first tetrapyrrole in the pathway, uroporphyrinogen III (Urogen), has been intensively studied (reviewed in Battersby and McDonald, 1979). Initially PBG was established as a precursor of tetrapyrroles in the green algae *Chlorella* (Bogorad and Granick, 1953) and in avian red blood cells (Falk *et al.*, 1953). The formation of uroporphyrinogen III from PBG requires the simultaneous presence of two enzymes, PBG deaminase and uroporphyrinogen III synthase (Bogorad, 1958a,b). There has been some controversy, mainly due to the chemical instability of the intermediate formed by PBG deaminase. However it is now accepted that PBG deaminase catalyses the condensation of four PBG units and the urogen synthase acts after the condensation has taken place. The condensation of the PBG units proceeds in the sequence ring A, B, C, D (Battersby *et al.*,1979a; Jordan and Seehra, 1979). The linear product is generally thought to be hydroxymethylbilane (Battersby *et al.*, 1983 a,b). Hydroxymethylbilane is unstable and spontaneously closes to form uroporphyrinogen I (a biologically inactive isomer). In the absence of deaminase, it has been shown that urogen synthase acts on the linear hydroxymethylbilane to produce the tetrapyrrole ring closure (with the required isomeric rearrangement) to form urogen in *Rb. sphaeroides* (Burton *et al.*,1979a,b; Jordan *et al.*, 1979) and in *Euglena gracilis* (Battersby *et al.*,1979b).

Porphobilinogen deaminase has been purified from a wide range of prokaryotes and eukaryotes, and has recently been crystallized in *E. coli* (Jordan *et al.*, 1988). Uroporphyrinogen III synthase has been purified to homogeneity from *Euglena gracilis* (Hart and Battersby, 1985) and human erythrocytes (Tsai *et al.*, 1987).

Recently two groups have isolated and sequenced the genes encoding the deaminase (*hem*C) and synthase (*hem*D) in *E. coli*. Initially the *E. coli hem*C gene was sequenced and found to encode a polypeptide of Mr=33,857 which agreed with the Mr=35,000 found on SDS-PAGE (Thomas and Jordan, 1986). A promoter consistent with a weakly transcribed *E. coli* gene along with a Shine-Dalgarno sequence were identified. An open reading frame which overlapped with the last codon of the *hem*C

open reading frame was also identified. Subsequently two groups sequenced the *hem*D gene of *E. coli* which was found to encode a polypeptide of Mr=27,887, similar to the Mr=29,000 obtained on SDS-PAGE (Sasarman *et al.*, 1987; Jordan *et al.*, 1987; Jordan *et al.*, 1988). They both confirmed that the start of the open reading frame of the *hem*D gene overlapped with the last codon of the *hem*C gene and agreed that *hem*C and *hem*D were linked in an operon transcribed from the *hem*C promoter. Both groups also identified a further open reading frame downstream of *hem*CD which lacked its own promoter. It is significant that *hem*G encoding a later haem enzyme protoporphyrinogen oxidase has been mapped in this area (Bachmann, 1983).

Two eukaryote full length cDNA clones bearing *hem*C, the gene encoding porpobilinogen deaminase, have been isolated and sequenced from human (Raich *et al.*, 1986) and rat (Stubnicer *et al.*, 1988). It has been shown in humans that there are two isoforms of this enzyme, one found in all cells and one found only in erythrocytes, which are both transcribed from the same gene but differ at their 5' ends (Chretien *et al.*, 1988).

1.2.4. Decarboxylation of uroporphyrinogen III to form coproporphyrinogen III

Uroporphyrinogen decarboxylase catalyses the sequential removal of four carboxyl groups of the carboxymethyl side chains of urogen to yield coproporphyrinogen III (coprogen) (Mauzerall and Granick, 1958; Battle and Grinstein, 1962; de Verneuil *et al.*, 1983). Extensive studies of its kinetic properties suggest that the decarboxylations might occur in a 2-stage process involving an initial rapid elimination of the first carboxyl group of the carboxymethyl of ring D, then the decarboxylation of the remaining three carboxyl groups in ring order A, B, and C at a slower rate (Tomio *et al.*, 1970). The enzyme has been purified from human erythrocytes, has a molecular weight of 46,000 and does not require any metal cofactors (de Verneuil *et al.*, 1983).

The *hem*E gene encoding uroporphyrinogen decarboxylase has been isolated from cDNA libraries of rat (Roméo *et al.*,1984) and human (Roméo *et al.*, 1986). Nucleotide sequence has been obtained for the full length cDNA of both rat (Romana *et al.*, 1987b) and human (Roméo *et al.*, 1986). The human *hem*E gene encodes a polypeptide of Mr=40,831 which is in good agreement with the value Mr=42,000 obtained on SDS-PAGE. Recently, the nucleotide sequence of the full length genomic human gene has been reported (Romana *et al.*, 1987a). Furthermore, the unique copy of the human *hem*E gene is transcribed from a single promoter and the level of enzyme activity, which shows tissue specific differences, is controlled at the level of transcription (Roméo *et al.*, 1986; Romana *et al.*, 1987a).

1.2.5. Oxidative decarboxylation of coproporphyrinogen III to form protoporphyrinogen I

Coproporphyrinogen III oxidase catalyses the oxidative decarboxylation of the 2- and 4- propionyl groups of coproporphyrinogen III (coprogen) into vinyl groups to form protoporphyrinogen IX (protogen) (Sano and Granick, 1961; Porra and Falk, 1964). A presumed intermediate of the reaction, containing three carboxyl groups was reported by Sano and Granick (1961), and subsequent work showed that the decarboxylation of ring A precedes that of ring B (Games *et al.*, 1976). In animals, coprogen oxidase is associated with mitochondria and molecular oxygen is required for activity (Sano and Granick, 196l; Porra and Falk, 1964; Grandchamp *et al.*, 1978). However in *Rb. sphaeroides* two forms of coprogen oxidase activity have been identified (Tait, 1969, 1972). Extracts from aerobically grown *Rb. sphaeroides* need only the presence of oxygen for coprogen oxidase activity. However extracts from anaerobically grown *Rb. sphaeroides* require Mg^{2+}, ATP, methionine and NAD^+ or $NADP^+$ for coprogen oxidase activity. Similar requirements were reported for anaerobic and aerobic coprogen oxidase activity in aerobic and anaerobically grown yeast cell extracts (Poulson and Polglase, 1974).

Yoshinaga and Sano (1980) purified coprogen oxidase from beef liver to homogeneity and found it to be a monomer (Mr=71,600). Recently, coprogen oxidase was purified to homogeneity in yeast, which shows a molecular weight of 35,000 (Camadro *et al.*, 1986). The coprogen oxidase was found to be a dimer, containing two iron atoms/molecule of protein and located in the cytosol. The enzyme was active only when molecular oxygen was used as an electron acceptor and had no anaerobic activity. However, it should be noted that haem is synthesised *in vivo* by anaerobically grown yeast cells (Zagorec and Labbe-Bois, 1986). The yeast coprogen oxidase was activated by phospholipids or neutral detergents as described previously for the bovine liver enzyme (Yoshinaga and Sana, 1980). This suggests that the enzyme may be membrane bound, and may reflect the role of coprogen oxidase in transporting protogen across the mitochondrial membrane as the next two enzymes involved in haem biosynthesis are reported to be located on the inner membrane of yeast mitochondria (Camadro *et al.*, 1986) and more recently using mouse mitochondria proposed to form an intrinsic membrane complex (Cruz Ferreira *et al.*, 1988). It is interesting to note that among the various reports of coprogen oxidase activity in plants, coprogen oxidase activity has been found mainly associated with mitochondria (Hsu and Miller, 1970). Since isolated chloroplasts are capable of forming protogen from precursors (Castlefranco *et al.*, 1979) it is indirectly assumed that chloroplasts contain a coprogen oxidase.

Recently, Zagorec *et al.*, (1988) used a yeast mutant with a defective coprogen oxidase activity to isolate *hem*13. *hem*13 has been sequenced and codes for a polypeptide of Mr=37,673. Transcripts for this gene were found to be more abundant in anaerobically grown wild type yeast cells. Addition of hemin to aerobic cultures lead to a substantial decrease in the level of the *hem*13 transcripts, showing that its expression is regulated negatively by haem and oxygen.

1.2.6. Dehydrogenation of protoporphyrinogen IX to protoporphyrin IX

The loss of six atoms of hydrogen to change protogen to protoporphyrin IX (proto), drastically changes the chemical properties of the pyrrole. Unlike protogen, proto has a rigid planar structure, which can chelate a wide variety of metallic ions and absorb light (Castlefranco and Beale, 1981). As the dehydrogenation of protogen can occur spontaneously, especially in the presence of molecular oxygen and light, no enzymatic involvement was initially thought necessary. However, specific enzymic catalysis of protogen was reported in *E. coli* (Jacobs and Jacobs, 1975; Poulsen *et al.*, 1976), yeast (Poulson and Polglase, 1975) and in *Rb. sphaeroides* (Jacobs and Jacobs, 1981).

The enzyme protoporphyrinogen oxidase has been partially purified in yeast (Poulson and Polglase, 1975), rat liver mitochondria (Poulsen, 1976) and barley mitochondria and etioplasts (Jacobs and Jacobs, 1987). Recently, a homologous preparation of protogen oxidase was reported from mouse liver mitochondria, where the enzyme showed a Mr=65,000 on SDS-PAGE (Dailey and Karr, 1987). Protogen oxidase seems to require no cofactors. This enzyme is bound to the inner mitochondrial membrane of eukaryotic cells, and in mammalian cells requires molecular oxygen (Poulson, 1976). In *E. coli*, Jacobs and Jacobs (1976) found that in the absence of oxygen an anaerobic electron acceptor such as fumarate could be utilized. In *Rb. sphaeroides* the full implications of the observation that respiratory inhibitors reduce the activity of protogen oxidase (Jacobs and Jacobs, 1981) need testing with homogenous enzyme preparations.

1.2.7. Formation of iron porphyrins

1.2.7.1 Synthesis of protohaem

The incorporation of Fe^{2+} into protoporphyrin IX is catalysed by ferrochelatase, a membrane associated enzyme. Ferrochelatase activity is high in the membranes of *Rb. sphaeroides* and its specificity has been examined in detail (Jones and Jones, 1970). Ferrochelatase has been purified to homogeneity from a variety of sources; rat

(Taketani and Tonkunaga, 1981), bovine (Dailey and Fleming, 1983), chicken erythrocytes (Hansone and Dailey, 1984) *Rb. sphaeroides* (Dailey, 1982; Dailey *et al.*, 1986) and yeast (Camadro and Labbe, 1988). Ferrochelatase purified from vertebrates has a Mr of around 40,000 on SDS-PAGE. The bacterial ferrochelatase from *Rb. sphaeroides* has a Mr of 105,000 on SDS-PAGE. The rat, chicken and yeast enzymes are activated by fatty acids, but the bovine enzyme is not. Using polyclonal antibodies raised against the purified yeast ferrochelatase Camadro and Labbe (1988) showed that ferrochelatase is synthesised as a Mr=44,000 precursor which is rapidly converted *in vivo* to a Mr=40,000 membrane bound form. It has been suggested that ferrochelatase forms part of a multi-enzyme complex (Camadro and Labbe, 1988; Cruz Ferreria *et al.*, 1988). In yeast mutants which lack protoporphyrinogen oxidase activity, although protoporphyrinogen IX is still produced by non-enzymic oxidation, ferrochelatase can not produce haem *in vivo* (Urban-Grimel and Labbe-Bois, 1981; Camadro *et al.*, 1982). This suggests that there may be some coupling mechanism between protophorphyrinogen oxidase activity and haem synthesis catalysed by ferrochelatase.

A common feature of all ferrochelatases is their inhibition by N-methylprotoporphyrin (DeMatteis *et al.*, 1980, Ortiz de Montellano *et al.*, 1980). In *Rb. sphaeroides*, addition of N-methylprotoporphyrin leads to a decrease in the level of intracellular haem. The consequent derepression of ALA synthatase produces an increase in protoporphyrin which is diverted to the bacteriochlorophyll pathway (Houghton *et al.*, 1982). All enzymes examined contain reactive sulfhydryl residues that are necessary for iron binding, and active arginyl residues involved in porphyrin binding (Dailey *et al.*, 1986; Dailey and Fleming, 1983).

1.2.7.2. The formation of haem a, haem c and siroheme

The origins of haems other than protohaem have been poorly studied. The little evidence available is reviewed in Rebeiz and Lascelles (1982). It is assumed that haem a and c are derived from protohaem and sirohaem is derived from urogen.

1.2.8. Protoporphyrin IX to Mg-protoporphyrin IX monomethyl ester

1.2.8.1. Mg chelation

The insertion of Mg into protoporphyrin was first shown by Gorchein (1972) using whole cells of *Rb. sphaeroides* made permeable to proto using EDTA. The product, Mg-protoporphyrin IX monomethyl ester (Mg-PME) suggested that the insertion of magnesium was tightly coupled to the methylation step (Gorchein, 1972). Mg

chelatase, the enzyme responsible for Mg insertion has not been purified to homogeniety. However in crude plastid extracts Mg chelatase seems stimulated by ATP (Pardo *et al.*, 1980). The observation that ATP is required for Mg chelation confirms earlier observations that Mg chelatase activity in whole *Rb. sphaeroides* cells decreases in the presence of inhibitors of electron transport (Gorchein, 1973).

1.2.8.2. The methylation of Mg-protoporphyrin IX

The methylation of Mg-protoporphyrin IX (Mg-proto) to Mg-protoporphyrin monomethyl ester (Mg-PME) is mediated by the enzyme s-adenosylmethionine-Mg-protoporphyrin methyl transferase, a reaction requiring s-adenosyl-methionine (SAM). Methyl transferase activity has been demonstrated in *Rb. sphaeroides* (Tait and Gibson, 1961; Gibson *et al.*, 1963), in *Euglena gracilis* chloroplasts (Ebbon and Tait, 1969) and in *Zea mays* chloroplasts (Radmer and Bogorad, 1967). In all cases the methyl transferase had a higher specifity for Mg-proto when compared to proto, thus establishing the sequence proto -> Mg-proto -> Mg-PME, two reactions which Gorchein (1972) proposed to be coupled. Richards and co-workers have used Mg-protoporphyrin affinity columns to purify methyl transferase from *Euglena gracilis* and wheat, and studied the enzyme kinetics in detail (Hinchigeri *et al.*, 1981). However both preparations contained four polypeptides with affinity for Mg-proto and there is still a need for a homogenous preparation.

1.2.9. Magnesium protoporphyrin monomethyl ester (Mg-PME) to Magnesium 2-vinyl, 4-ethyl pheoporphyrin a5 monomethyl ester (PChlide)

The conversion of Mg-PME to PChlide requires the modification of the tetrapyrrole side chains at positions 4 and 6. Although there is some debate as to the sequence of reactions, it is generally accepted that the formation of the cyclopentane ring from the methyl propionate side chain at position 6 precedes the hydrogenation of the vinyl group at position 4 (Castefranco and Beale, 1981). The knowledge of the sequence between Mg-PME and bchl/chlorophyll formation is incomplete, due to the mixtures of tetrapyrroles observed. It has been proposed that many parallel pathways exist in higher plants (Rebeiz and Lascelles, 1982; Tripathy and Rebeiz, 1986), and to some extent in *Rb. sphaeroides* (Pudek and Richards, 1975).

Much of the early work establishing the intermediates of the reactions from Mg-PME, have involved study of photosynthetic bacteria, particularly *Rb. sphaeroides* and *Rb. sphaeroides* mutants which accumulate tetrapyrrole intermediates. Magnesium 2, 4-divinyl pheoporphyrin a5 monomethyl ester (DVPChlide) was first identified by Jones (1963 a,b) when *Rb. sphaeroides* was treated with 8-

hydroxyquinoline. DVPChlide was also identified as the major pigment in the inner seed coat of *Cucubita pepo* (Jones, 1966). Mutants of R*b. sphaeroides* which excrete DVPChlide have been isolated (Lascelles, 1966a; Richards and Lascelles, 1969). The DVPChlide excreted by a *Rb. sphaeroides* mutant was converted into a pigment similar to chlorophyllide by barley etioplasts (Griffiths and Jones, 1975). The enzyme involved in cyclopentanone ring formation has been detected in the etioplasts of cucumber and called magnesium protoporphyrin IX monomethyl ester oxidative cyclase (Chereskin *et al.*,1982). Molecular O_2 was found to be required for Mg-PME oxidative cyclase activity (Chereskin *et al.*, 1982). Recently two mutants of *Rb. sphaeroides* which excrete Mg-PME and DVPChlide respectively have been used to look at the Mg-PME oxidative cyclase enzyme in wheat etioplasts using a continuous assay method (Nasrulhaq-Boyce *et al.*, 1987). It was concluded that oxidative cyclase activity occurred only in intact etioplasts and requires NADPH and O_2, and the involvement of Fe^{2+}. Neither the oxidative cyclase or the enzyme involved in the hydrogenation reaction have been purified.

1.2.10. Magnesium 2-vinyl, 4-ethyl pheoporphyrin a5 monomethyl ester (PChlide) to Chlorophyllide a (Chlide)

The conversion of PChlide to Chlide involves the reduction of ring D, when two hydrogens are added in trans at the double bond between carbons 7 and 8. In plants the change of PChlide to Chlide is mediated by a light dependent NADPH-protochlorophyllide reductase. Angiosperms are unable to form chloroplasts in the dark. When a seedling is grown in the dark the protoplasts in developing leaves are transformed into etioplasts. The etioplast lacks chlorophyll and possesses no extensive thylakoid membrane. When etiolated leaves are exposed to light PChlide is rapidly converted to Chlide (Shibata, 1957). Photoactive PChlide-protein complexes have been isolated from etiolated tissues. The smallest isolated PChlide-protein is probably an enzyme-substrate complex of NADPH-PChlide reductase (Castlefranco and Beale, 1983). On illumination PChlide reductase activity decreases rapidly (Mapleston and Griffiths, 1980; Santel and Apel, 1981) and coincides with a decrease in the amount of enzyme protein (Santel and Apel, 1981; Oliver and Griffiths, 1984). Further study has shown that the light induced decrease in PChlide reductase also coincides with a reduction in the level of specific mRNA which is translatable (Apel, 1981).

Kay and Griffiths (1983) showed that the light induced breakdown in vitro can be prevented by the addition of PChlide and NADPH. They concluded that the breakdown of PChlide reductase on illumination was the result of the production of a substrate free enzyme on release of Chlide after the photochemical reduction of

PChlide. Apel and co-workers have used purified polyclonal antibodies raised against the 36,000 molecular weight PChlide reductase to look at the compartmentalisation of the enzyme in barley. They found that in broken plastids the level of the PChlide reductase protein decreases more rapidly on illumination when compared to unbroken plastids. Furthermore, the PChlide reductase protein appeared even more stable after prolonged periods of illumination in intact leaves compared with intact plastids (Dehesh *et al.*, 1986a). Immuno-gold labelling of sections of etiolated and illuminated barley leaves showed two populations of immunoreactive protein, plastid and plasmalemma. The plastid polypeptide declined rapidly on illumination whereas the level of extraplastic polypeptide was stable (Dehesh *et al.*,1986b). The presence within the plastid compartment of proteolytic activities, which are highly specific for NADPH-PChlide reductase (Kay and Griffiths, 1983; Häuser *et al.*, 1984) makes it seem likely that the
light induced decline of the plastid-localized enzyme protein is due to a proteolytic breakdown. The role of the extraplastic protein in not understood (Dehesh *et al.*, 1986b).

Most gymnosperms and lower plants produce chlorophyll in the dark. There is evidence that these organisms contain two pathways, one with a light activated PChlide reductase, and the other with a unstudied enzyme sequence (for review see Castlefranco and Beale, 1981). In *Rb. sphaeroides* no mutant which accumulates PChlide has been identified and consequently this step has not been subjected to the same level of study. *Rb. sphaeroides* produces bacteriochlorophyll when grown anaerobically in the dark (Cohen-Bazire *et al.*, 1957) and therefore does not show the light dependent PChlide reductase activity shown in angiosperms.

1.2.11. Chlorophyllide a to chlorophyll a in plants

Pchlide is esterified at the propionic acid side chain to phytol to give chlorophyll *a*. Rüdiger and co-workers have established that chlorophyllide *a* is first esterified by geranyl-geranyl pyrophosphate (Rüdiger *et al.*, 1980) then three of the four double bonds of this ester are sequentially reduced to form the phytyl residue of chlorophyll *a* (Benz and Rüdiger, 1981). Chlorophyllase, though widely proposed to be active in this reaction, is thought to be a degradative enzyme since it cannot catalyse the esterification of Chlide and geranyl-geranylpyrophosphate (reviewed by Porra and Meisch, 1984). The enzyme(s) involved in this reaction remain elusive.

1.2.12. The formation of bacteriochlorophyll a from chlorophyllide in *Rb. sphaeroides*

The pathway from chlorophyllide *a* to bacteriochlorophyll *a* has been formulated from the study of mutants of *Rb. sphaeroides* which excrete tetrapyrrole intermediates (Lascelles, 1966a; Richards and Lascelles, 1969; Pudek and Richards, 1975). The order of the first two reactions appears interchangeable (Pudek and Richards, 1975). One reaction leads to the hydration of the vinyl group at position 2. The intermediate accumulated by *Rb. sphaeroides* mutants blocked in this step is 2-Desacetyl-2-vinyl bacteriochlorophyllide *a* (Pudek and Richards, 1975). The other reaction involves the reduction of ring B, when two hydrogens are added in trans across the double bond between carbons 3 and 4. The intermediate accumulated by *Rb.sphaeroides* mutants blocked in this step is 2-Devinyl, 2-hydroxyethylchlorophyllide *a* (Lascelles, 1966a; Richards and Lascelles, 1969). The third step in the reaction sequence involves the dehydrogenation of the hydroxyethyl group at position 2.
A *Rb. sphaeroides* mutant was isolated which excreted an intermediate identified as 2-Deacetyl-2-hydroxyethyl bacteriochlorophyllide *a* characteristic of a block in this reaction (Richards and Lascelles, 1969). The final step of bacteriochlorophyll biosynthesis is the addition of phytol to the propionic acid side chain at position 7 to convert bacteriochlorophyllide *a* to bacteriochlorophyll *a*. Since bacteriochlorophyllide *a* excreting mutants have been found (Richards and Lascelles, 1969) it seems that the phytylation step is the last step in the biosynthesis of bacteriochlorophyll *a* (mechanistically examined in Emery and Akhtar, 1987)

None of the enzymes involved in the conversion of Chlide to bacteriochlorophyll (bchl) have been purified or studied in *in vitro* systems. Although other similar mutants in bacteriochlorophyll biosynthesis have been described in other members of the *Rhodospirillaceae* family (see Saunders, 1978 for review), the work done by Lascelles and Richards on *Rb. sphaeroides* was most conclusive. In the closely related bacterium *Rhodobacter capsulatus*, Marrs and co-workers have assigned genes to the specific lesions on a pathway based on the studies of Lascelles and Richards (Biel and Marrs, 1983). The work on *Rb. capsulatus* will be described in more detail later with reference to the molecular genetic techniques employed. The overall regulation of bacteriochlorophyll biosynthesis will be considered in depth along with photosynthetic membrane development.

1.3. Molecular genetic techniques in *Rhodospirillaceae*

Rhodospirillaceae, especially *Rb. sphaeroides*, have been widely favoured for the study of many aspects of photosynthesis, as their ability to grow chemoheterotrophically allows the study of mutations affecting the photosynthetic apparatus (Saunders, 1978). For the past fifteen years application of new molecular techniques in *Rhodospirillaceae* particularly *Rb. capsulatus* and *Rb. sphaeroides* has made this group of photosynthetic bacteria a leading 'model system' for photosynthetic research. The range of molecular techniques used in *Rhodospirillaceae* has been reviewed recently by Scolnik and Marrs (1987).

1.3.1. The genetic mapping of the photosynthetic gene cluster of *Rb. capsulatus*

An unusual method of genetic exchange mediated by phage-like particles called gene transfer agents (GTA) was found in many wild type strains of *Rb. capsulatus* (Marrs, 1974, 1978b). Gene transfer mediated by GTA was used to map a region of the *Rb. capsulatus* chromosome carrying genes for carotenoid and bacteriochlorophyll biosynthesis (Yen and Marrs, 1976). Initially, seven clusters of mutations, five affecting carotenoid biosynthesis and two affecting bacteriochlorophyll biosynthesis, were arranged in one linkage group (Yen and Marrs, 1976) (Figure 1.5A). A further carotenoid lesion, *crtF* was positioned on the *Rb. capsulatus* genetic map using the same techniques (Scolnik *et al.*, 1980) (Figure 1.5B). However the use of GTA was limiting as only small amounts of DNA (around 4.6 kb) could be transferred and therefore only genes lying close together could be mapped using this technique. Recently, the GTA system has been used to introduce interposon insertions into *Rb. capsulatus* nitrogenase (*nif*) and carotenoid (*crt*) genes (Scolnik and Haselkorn, 1984; Giuliano *et al.*, 1988). *Rb. capsulatus* is the only bacterium in which this, unusual method of gene transfer has been observed.

Promiscuous R plasmids have been used in *Rb. sphaeroides* and *Rb. capsulatus* to establish chromosome transfer (Miller and Kaplan, 1978; Marrs, 1981; Sistrom *et al.*, 1984; Willison *et al.*, 1985) . Marrs (1981) isolated a number of R-prime plasmids bearing *Rb. capsulatus* chromosomal DNA which complemented all available mutants in bacteriochlorophyll and carotenoid biosynthesis, and in reaction centre synthesis. In *Rb. sphaeroides* a similar R-prime plasmid pWS2 was reported to bear a number of genes involved in bacteriochlorophyll and carotenoid biosynthesis, and those encoding light harvesting and reaction centre polypeptides (Sistrom *et al.*, 1984). However the data to substantiate this claim has not been presented. Pemberton and coworkers used an RPl derivative to develop a linkage map of the *Rb. sphaeroides* chromosome which placed six different carotenoid and two different bchl

lesions between the nitrogen fixation gene *nif*-1 and the adenosine biosynthesis gene *ade*-3 (Pemberton and Bowen, 1981; Bowen and Pemberton, 1985) (figure 1.6). Furthermore, Pemberton and Harding (1986) isolated two overlapping cosmids and confirmed the linkage of the six carotenoid genes in a 15 kb cluster in *Rb. sphaeroides* (figure 1.6).

The 50 kb fragment of *Rb. capsulatus* chromosomal DNA carried on the R-prime plasmid pRPS404 was analysed by restriction mapping and subcloned into kanamycin resistant derivative of pBR322 (Taylor *et al.*, 1983). Recombinant plasmids bearing various restriction fragments were mobilized into a variety of *Rb. capsulatus* mutants. The positions of a number of lesions in seven bacteriochlorophyll and six carotenoid biosynthesis genes were presented in a map bounded by the *rxc*B and *rxc*A genes which were identified as important for reaction centre synthesis. The physical dimensions of the genetic map agreed with those obtained using the GTA system (Figure 1.5C). It should be noted that *rxc*B and *rxc*A have been renamed *puh* and *puf* respectively (Kaplan and Marrs, 1986). The genetic-physical map of the *Rb. capsulatus* chromosome was extended using transposon Tn5.7 mutagenesis and complementation analysis of the various pRPS404::Tn5.7 plasmids (Zsebo and Hearst, 1984). Initially, 45 transposon Tn5.7 insertions were mapped on the *Rb. capsulatus* photosynthetic gene cluster. Four new *bch* genes, two new *crt* genes and a gene affecting reaction centre synthesis were identified and positioned. Three operons were described and the direction of transcription in each case identified (Figure 1.5D). The existence of the *crt*I gene first identified by Zsebo and Hearst (1984), was confirmed with the localisation of two point mutations which produced the *crt*I phenotype in the same region of the *Rb. capsulatus* photosynthetic cluster (Giuliano *et al.*, 1986).

Scolnik and co-workers have fine-mapped the *crt* cluster of *Rb. capsulatus* using interposon mutagenesis (Giuliano *et al.*, 1988). Fifteen interposon insertions defined seven carotenoid genes in a 8 kb cluster, within the photosynthesis region of *Rb. capsulatus*, which was flanked by genes affecting bacteriochlorophyll synthesis (see figure 1.5E). All the *crt* genes with the exception of *crt*I and *crt*B were organised in single transcriptional units. As each mutant is created at a premapped restriction endonuclease site, interposon mutagenesis revealed the need to revise the maps produced previously (Taylor *et al.*,1983; Zsebo and Hearst, 1984).

1.4. Construction of a pathway for carotenoid biosynthesis in *Rb. capsulatus*

Carotenoids have an important role in the photosynthetic membrane of *Rhodospirillaceae*, forming an integral part of the membrane bound light harvesting and reaction centre complexes. The presence of carotenoid seems to be essential for the stable integration of LHII into photosynthetic membranes as carotenoidless mutants of *Rb. sphaeroides* (Cogdell and Crofts, 1978; Lommen and Takemoto, 1978) and *Rb. capsulatus* (Zsebo and Hearst,1984) lack the LHII light harvesting complex. Carotenoids are also involved in capturing light energy within the light harvesting complexes (reviewed in Hunter *et al.*, 1988a). Probably an equally important role of carotenoids is the protection against photooxidation. In high light conditions chlorophylls (and many porphyrins) can produce oxygen radicals, which in the absence of carotenoids, produce fatal photooxidative damage (Anderson and Robertson, 1960).

Several possible routes for carotenoid biosynthesis have been proposed from biochemical studies (Schmidt, 1978; Goodwin, 1980). Recently, schemes for this pathway based on carotenoid synthesized by mutants in *Rb. capsulatus* and a number of higher plants have been proposed. Scolnik initially suggested a pathway based on carotenoid intermediates synthesised by mutants of *Rb. capsulatus* (Scolnik *et al.*, 1980). This pathway was later extended with the addition of the *crtI* gene product (Giuliano *et al.*, 1986; Giuliano *et al.*, 1988)(Figure 1.7). The formation of geranyl geranyl pyrophosphate (GGPP), a precursor of carotenoid and the phytol moiety of chlorophyll, from acetyl CoA is reviewed by Goodwin (1980). A number of attempts have been made to delineate a similar pathway in higher plants using a variety of carotenoid mutants (Thelander *et al.*, 1986; Scolnik *et al.*, 1986; Scolnik *et al.*, 1987). There is a degree of similarity between plant and *Rb. capsulatus* derived pathways particularly the steps up to neurosporene formation (Scolnik *et al.*, 1986). It is interesting to note that carotenoid less mutants of maize (Mayfield and Taylor, 1984; Mayfield *et al.*, 1986) and barley (Batshauer *et al.*, 1986) have no light harvesting chlorophyll a/b protein (LHCP).

In plants the hormone abscisic acid has a number of roles in development including, bud and seed dormancy, elongation of growth, stomatal opening, root growth, geotrophism, fruit ripening and scenescence (Scolnik,1987). It has been postulated that abscisic acid is made by two pathways, indirect and direct (Milborrow, 1983; Scolnik, 1987). The indirect pathway involves the formation of abscisic acid from the carotenoid zeaxanthin. From the involvement of carotenoids in abscisic acid and LHCP biosynthesis it would seem that carotenoids play a extremely important role in plant development.

1.5. The organisation of the bacterial photosynthetic membrane

The structure and composition of the photosynthetic membranes in *Rhodospirillaceae* have been studied using a number of biophysical and biochemical techniques (Kaplan and Arntzen, 1982; Drews, 1985; Zuber, 1986; Zuber, 1987; Hunter *et al.*, 1988a). The photosynthetic apparatus is located on specialised invaginations of the cytoplasmic membrane (CM) called the intracytoplasmic membrane system (ICM) which extend into the interior of the cell (Niederman and Gibson, 1978). The ICM is rich in pigment binding proteins, which in *Rhodospirillaceae* consist of one or two light harvesting pigment protein complexes called LHI and LHII, which surround and interconnect reaction centres (RC). The number of light harvesting complexes and their relationships to each other has been studied particularly in relationship to energy transfer (Pearlstein, 1982; van Grondelle, 1985; Hunter *et al.*, 1988a). Singlet-triplet annihilation studies (Monger and Parson, 1977) have suggested that LHI, which acts as a core antenna, surrounds and interconnects reaction centres and that 'lakes' of LHII are arranged around this core network (Figure 1.8A). On the basis of low temperature singlet-singlet annihilation data this model was refined so that four reaction centres are thought to be surrounded by 12 to 18 LHI complexes, which are arranged around and between the reaction centres (Vos *et al.*, 1986; Hunter *et al.*, 1988a).

1.5.1. The light harvesting complexes

A number of groups have used *Rhodospirillaceae* as the source of preparations of light harvesting complexes after solubilising membranes with various detergents (Clayton and Clayton, 1972; Cogdell and Crofts, 1978; Broglie *et al.*, 1980) reviewed in Zuber (1985). The isolation of complexes showed that the three characteristic absorption peaks 800nm, 850nm and 875nm in *Rb. sphaeroides* were due to two different complexes LHI (also called B875 after its absorbency maximum in the near infrared) and LHII (also called B800-850) (Clayton and Clayton, 1972; Broglie *et al*; 1980). Each complex is made up of equimolar amounts of two polypeptides (50 to 60 amino acids long) called α and ß. All four polypeptides involved in light harvesting have several features in common (Theiler *et al.*, 1984; 1985; Zuber, 1987).

(a) Each has three distinct domains, hydrophilic carboxyl- and amino-terminal regions, separated by 20 to 30 amino acids which form a membrane spanning α helix.

(b) One or two conserved histidines thought to bind bacteriochlorophyll.

The LHI complex of *Rb. sphaeroides* consists of LHI ∝ and LHI ß polypeptides in equimolar amounts, and pigment ratios of 1 bchl : 1 carotenoid (Broglie *et al.*, 1980). The molecular weights of the LHI∝ and LHI ß polypeptides have been determined as 6,809 daltons and 5,457 daltons respectively from amino acid sequencing (Theiler *et al.*, 1985). The electrophoretic mobility of isolated LHI complexes suggests a minimal unit of 3∝ and 3ß polypeptides with 6 bch1 875 molecules and 6 carotenoids (Hunter *et al.*, 1988a)(see figure 1.8B).

The LHII complex of *Rb. sphaeroides* consists of equimolar amounts of LHII∝ and LHIIß polypeptides with a pigment ratio of 2 bchl : 1 carotenoid (Broglie *et al.*, 1980; Hunter *et al.*, 1988a). The molecular weights of LHII∝ and LHIIß have been determined from amino acid sequence data as 5,599 and 5,448 daltons respectively (Theiler *et al.*, 1984). The LHII complex is thought to contain 3∝ and 3ß polypeptides, 6 Bchl 850, 3 Bchl 800 and 6 carotenoid molecules (Hunter *et al.*, 1988a)(Figure 1.8C). However the structure of both the LHI and LHII complexes await determination by X-ray crystallographic techniques.

1.5.2. The reaction centre

The reaction centre complex of *Rb. sphaeroides* consists of three polypeptides RC-H, RC-M and RC-L (heavy, medium and light based on their relative mobilities in SDS-PAGE) in a 1 : 1 : 1 stoichiometric ratio with 4 molecules of bchl, 2 molecules of bacteriopheophytin (bphe), 2 molecules of quinone, a non haem iron and a single carotenoid (Feher and Okamura, 1978). From nucleotide sequence data the molecular weights of RC-H, RC-M and RC-L are 28,003, 34,265 and 31,319 respectively (Williams *et al.*, 1983, 1984, 1986). The RC complexes of both *Rhodopseudomonas viridis* and *Rb. sphaeroides* have been crystalised and the structure of the complex determined to a resolution of 3Å and 2.8Å respectively (Deisenhofer *et al.*, 1985; Michel *et al.*, 1986; Allen *et al.*, 1987a,b; Yeates *et al.*, 1987). The X-ray crystal structure analysis shows remarkable similarity between these species. Both L and M subunits contain 5 transmembrane helixes, which bind between them 4 bchl (2 forming the'special pair'), 2 bphe, 2 quinones and one non haem iron (reviewed by Knaff, 1988). The similarity between RC-L and RC- M, and Dl and D2 polypeptides of higher plant reaction centres is pronounced (reviewed by Michel and Deisenhofer, 1988). Although RC-L and RC-M are involved in primary charge separation, the function of RC-H is not understood. The availability of the crystal structure allows site- directed mutagenesis techniques to be used to probe the structure-function relationship of each amino acid. Bylina and Youvan (1987) have used site directed mutagenesis to change isoleucine 229 of RC-L to seventeen other amino acids, six of which resulted in resistance to the herbicide atrazine.

1.6. Analysis of genes encoding components of the photosynthetic membrane

1.6.1. The *puf* operon

The *puf* operon encodes four identified polypeptides; LHI∝and ß, RC-L and RC-M. Initially Williams and co-workers used oligonucleotide probes based on amino terminal sequences of the reaction centre L and M polypeptides of *Rb. sphaeroides* to isolate clones. DNA sequence analysis demonstrated genes for RC-L and RC-M were linked in the same operon (Williams *et al.*, 1983,1984). They also pointed out that L and M showed homology with each other and the *psb*A gene of higher plants encoding the plant thylokoid protein Dl, now known to be a component of the photosystem II reaction centre (reviewed in Michel and Deisenhofer, 1988). At the same time Youvan and co-workers isolated a clone from the 46 kb photosynthetic cluster of Rb. *capsulatus* which complemented mutants lacking reaction centre and LHI polypeptides. A 4.8 kb *Eco* RI - *Bam* HI fragment was sequenced and found to encode LHI∝and ß, and RC-L and RC-M polypeptides linked in an operon, now called *puf*, in the order LHIß-LHI∝-RCL-RCM (Youvan *et al.*, 1984). It should be noted that the nomenclature *puf*B-*puf*A-*puf*L-*puf*M is now used (Kaplan and Marrs, 1986). The L and M sequences were again found to be homologous with each other and the Dl polypeptide of higher plants. Two groups have extended the nucleotide sequence of the *puf* operon of *Rb. sphaeroides* to include the genes encoding LHI∝ and ß (Kiley *et al.*, 1987; Ashby, 1988). The nucleotide sequence of genes encoding L and M subunits of *Rhodopseudomonas viridis* has also been obtained (Michel *et al.*, 1986). Recently, the nucleotide sequence of a third *puf* operon from *Rhodospirillum rubrum* has been obtained (Belanger *et al.*, 1988; Belanger and Gringas, 1988). The three *puf* operons *Rb. sphaeroides*, *Rb. capsulatus* and *Rs. rubrum* show complete identity in gene order, *puf*BALM, and close similarity in size.

In the search for the oxygen regulated promoter of the *puf* operon the nucleotide sequence was extended upstream of *puf*B. An open reading frame designated *puf*Q was found that could encode a polypeptide 74 amino acids in length and showed a codon usage consistent with that for other genes from this bacteria (Bauer *et al.*, 1988). When *puf*Q was deleted along with the downstream region of the *puf* operon 60-fold less bacteriochlorophyll was synthesised, along with correspondingly low amounts of LHII. The same deletion in bchl$^-$ mutants of *Rb. capsulatus* lead to a substantial decrease in the excretion of intermediates. Furthermore, a construction was made in which a *puf-lacZ* gene fusion was placed under control of the *nif* HDK promoter. This construction showed that when expression from the *nif* HDK promoter was induced under various nitrogen conditions the amount of bchl synthesis was directly proportional to the amount of *puf*Q synthesised (measured as ß-galactosidase activity). Bauer and Marrs suggest that the *puf*Q gene product is

essential for bacteriochlorophyll biosynthesis (Bauer and Marrs, 1988). It has been reported that intermediates of bchl synthesis formed insoluble tetrapyrrole/protein/lipid complexes (Lascelles, 1966a; Drews, 1974; Richards *et al.*, 1975) that are associated with a small (9,000 dalton) hydrophobic protein (Richards *et al.*, 1975). Lascelles (1966a) was the first to propose that bchl biosynthesis could be controlled by regulating the synthesis or activity of a membrane associated polypeptide. An open reading frame which shows strong homology to *puf*Q of *Rb. capsulatus* has been identified upstream of the *Rb. sphaeroides puf*B gene (Ashby, 1988). Deletion of the *puf* operon including *puf*Q in *Rb. sphaeroides* also produced a cessation of bchl synthesis along with the accumulation of a bacteriochlorophyll intermediate with an absorbance peak at 640nm. Furthermore, Davis *et al.*, (1988) deleted part of the *puf*Q region of *Rb. sphaeroides* and inserted kanamycin resistance gene cartridge. The resulting puf$^-$ strain showed reduced bchl synthesis and a consequent low level of LHII. It is clear from the results so far gained that the *puf*Q gene product is essential for bacteriochlorophyll biosynthesis.

1.6.2. The *puh*A gene

Two groups have cloned and sequenced *puh*A, the gene encoding the reaction centre H subunit in *Rb. capsulatus* (Youvan *et al.*, 1984) and in *Rb. sphaeroides* (Williams *et al.*, 1986). In *Rb. capsulatus* the *puh*A and *puf* genes form the limits of the photosynthesis gene cluster (Youvan *et al.*, 1984) (Figure 1.5D).

1.6.3. The *puc* operon

The *puc* operon encodes genes for the light harvesting LHIIα and ß polypeptides. Youvan and Ismail (1985) first sequenced the *puc* operon which was isolated using oligonucleotides based on the amino terminal sequences of LHIIα and ß. Using similar methods the *puc* operon was isolated and sequenced in *Rb. sphaeroides* (Ashby *et al.*, 1987; Kiley and Kaplan, 1987). In *Rb. capsulatus* and *Rb. sphaeroides* both operons are of similar size and gene order.

1.6.4. The *pet* operon

The genes for the membrane bound cytochrome b/c, complex, which is involved in photosynthetic electron transport have been cloned and sequenced in *Rb. capsulatus* (Gabellini *et al.*, 1985; Gabellini and Selbald, 1986; Daldal *et al.*, 1987; Davidson and Daldal, 1987a). It should be noted that Gabellini and co-workers believed they were

working on *Rb. sphaeroides* which unfortunately turned out to be *Rb. capsulatus* (Davidson and Daldal, 1987b). The gene order for the pet operon is *pet*A-*pet*B-*pet*C.

1.6.5. The *cyc*A gene

The gene encoding the extrinsic protein cytochrome c_2 involved in light driven electron transport has been named *cyc*A. *cyc*A has been cloned and sequenced in both *Rb. capsulatus* (Daldal *et al.*, 1986) and *Rb. sphaeroides* (Dohohue *et al.*, 1986b).

1.6.6. Pigment biosynthesis genes

Although the genes for carotenoid and bchl biosynthesis have been cloned and characterized on the photosynthetic gene cluster of *Rb. capsulatus* (figure 1.5), no nucleotide sequence has been published.

1.7. Regulation of photosynthetic membrane assembly

Cohen-Bazire *et al.*, (1957) were the first to observe that light and oxygen affected the concentration of bchl in *Rhodospirillaceae*. At concentrations of O_2 of less than 2% the cell develops an extensive system of intracellular membranes that carries large amounts of photosynthetic pigment-protein complexes. Furthermore, the amount of intracellular membrane per cell and the pigment-protein composition are also affected by light intensity. Therefore the synthesis and composition of the intracellular membranes are under at least two different levels of control, oxygen concentrations and light intensity (reviewed by Kaplan and Arntzen, 1982; Kiley and Kaplan, 1988). The regulation of photosynthetic membrane assembly has been looked at in a variety of ways at all levels of control, including transcription and translation, in both *Rb. capsulatus* and *Rb. sphaeroides*. However, the variety of conditions and environmental shifts that have been used, particularly in work on mRNA transcript levels, has made comparisons between different studies difficult. Three principle systems are regularly utilized.

1. Shifts involving only one known effector: eg. high to low oxygen concentration or high to low light intensity.

2. Shifts involving more than one known effector:eg. phototrophic (anaerobic, light) to chemotrophic (semiaerobic/dark) growth shifts.

3. Steady state environment: eg. photosynthetic (anaerobic,light), chemotrophic (semiaerobic, dark). However, chemostats are rarely used to produce controlled growth conditions.

1.7.1. Regulation of expression of the *puf* operon

The *puf* operon encodes LHIα and ß, RC-L and RC-M polypeptides. All three *puf* operons are reported to have two major transcripts, a 0.6-0.5 kb transcript which encodes the LHIα and ß polypeptides, and a 2.6-2.7 kb transcript which encodes LHI ß and RC-LM polypeptides (in *Rb. capsulatus*; Belaso *et al.*, 1985; Zhu and Hearst,1986; Zhu *et al.*, 1986a, in *Rb. sphaeroides*; Zhu and Kaplan, 1985, Zhu *et al.*, 1986b; Hunter *et al.*, 1987, and in *Rs. rubrum*; Belanger and Gingras, 1988). It was quickly noted that the stability of the two transcripts differed. The half life of the longer transcript was found to be 4-10 minutes compared with 20 minutes for the 0.5 kb transcript (Belasco *et al.*, 1985; Zhu *et al.*, 1986a; Belanger and Gingras, 1988). Intercistronic stem loop structures between *puf*A and L have been found to account for the differences in transcript stability. Deletion of this structure in *Rb. capsulatus* has been found to destabilise the 0.5 kb mRNA (Klug *et al.*, 1987; Chen *et al.*, 1988). Chen *et al.*, (1988) suggested that the decay of the 3' segment begins with endonucleolytic cleavage.

From comparisons of mRNA transcript levels it is clear that the level of mRNA transcripts originating from the *puf* operon is regulated by oxygen in both *Rb. capsulatus* (Clark *et al.*, 1984; Klug *et al.*, 1985; Zhu and Hearst, 1986; Zhu *et al.*, 1986a) and in *Rb. sphaeroides* (Zhu and Kaplan, 1985; Hunter *et al.*, 1987; Zhu *et al.*, 1986b). In *Rb. capsulatus* a shift from anaerobic to aerobic conditions produces a rapid decrease in the levels of both *puf* transcripts (Zhu *et al.*, 1986a). It has also been recorded that the presence of oxygen reduces the half lives of both *puf* transcripts by half in both *Rb. sphaeroides* (Zhu *et al.*, 1986b) and *Rb. capsulatus* (Zhu *et al.*, 1986a). When a shift from aerobic to anaerobic growth occurs the level of specific transcripts from the *puf* operon increases 40-fold in *Rb. capsulatus* (Clark *et al.*, 1984) with an equivalent stimulation found in *Rb. sphaeroides* (Hunter *et al.*, 1987). Comparison between steady state levels of *Rb. capsulatus puf* transcripts from high light ($100W/m^{-2}$) and low light ($3W/m^{-2}$) growth conditions revealed that in low light transcript levels were only 1.3 to 1.7 fold higher (Zhu and Hearst, 1986). A similar comparison of steady state transcript levels in *Rb. sphaeroides* also revealed *puf* transcript levels were 1.5 to 1.8 fold higher in low light conditions (Zhu and Kaplan, 1985). In *Rb. capsulatus* the ratio of 0.5 kb transcript (*puf*BA) to the 2.6 kb transcript (*puf*BALM) is 10 to 1. It has been proposed that this accounts in part for the

stoichiometric ratios of LHI to RC complexes of 12-18 to 1 (Belasco *et al.*, 1985; Kaufman *et al.*, 1982).

1.7.2. Regulation of expression of the *puh*A operon

The *puh*A operon encodes the RC-H polypeptide. In *Rb. capsulatus puh*A encodes two transcripts of 1.2 and 1.4 kb (Zhu and Hearst, 1986). Similar sized transcripts of 1.1 and 1.4 kb have also been found in *Rb. sphaeroides* (Dohohue *et al.*, 1986a). In *Rb. capsulatus* fluctuation in the level of the *puh*A transcripts in different light and oxygen conditions is very similar to that described for the *puf* operon (Zhu *et al.*, 1986a; Zhu and Hearst, 1986).

1.7.3. Regulation of expression of the *puc* operon

The *puc* operon encodes LHII∝ and ß polypeptides. In *Rb. capsulatus* the transcript length is 0.5 kb (Zhu and Hearst, 1986; Zhu *et al.*, 1986a). Similarly in *Rb. sphaeroides* a *puc* transcript of 0.55 - 0.65 kb has been described (Kiley and Kaplan, 1987; Hunter *et al.*, 1987). When *Rb. capsulatus* undergos a shift from anaerobic to aerobic growth the level of the 0.5 kb transcript diminishes rapidly (Zhu *et al.*, 1986a). The *puc* transcript is relatively stable with a half life of 20.5 minutes in *Rb. sphaeroides* (Kiley and Kaplan, 1988). On shifting from aerobic to anaerobic growth, the maximum level of transcripts encoding *puc* lagged that of *puf* by 25 minutes, which corresponds with a similar delay in forming LHII complex compared to LHI and RC complexes in *Rb. capsulatus* (Klug *et al.*, 1985). In *Rb. sphaeroides* the rise in the level of transcripts specific for *puc* genes was also found to lag behind that of *puf* (Hunter *et al.*, 1987), supporting observations that the LHII complex is produced latter than LHI/RC core complexes in photosynthetic membrane development (Niederman *et al.*, 1976; Hunter *et al.*, 1982).

1.7.4. Regulation of expression of pigment biosynthesis genes

It has been known for a long time that the bacteriochlorophyll content of cultures of *Rhodospirillaceae* is regulated by light and oxygen (Cohen-Bazire *et al.*, 1957; Arnheim and Oelze, 1983b). It was found that although both light and oxygen controlled bchl levels, only oxygen affected the level of 5-aminolevulinate synthase activity in *Rb. sphaeroides* (Oelze and Arnheim, 1983). This led to the suggestion that oxygen and light act independently of each other (Arnheim and Oelze, 1983a,b).

Although none of the pigment biosynthesis genes have been sequenced some work has been performed to investigate transcriptional regulation. Biel and Marrs (1983)

examined the regulation at the level of transcription using Mu dl (Ap^R lac) phage insertions into *bch* genes. This study revealed for the first time that the bacteriochlorophyll biosynthesis genes *bch*B, *bch*C, *bch*G and *bch*H were regulated by oxygen but not by light. ß-galactosidase activity presumed under the control of these *bch* promoters increased 2 to 3.5 fold in response to lowering the oxygen concentration (20% to 1-2%).

A number of studies have been aimed at looking at levels of mRNA encoding *bch* and *crt* genes from the pigment biosynthesis region of the *Rb. capsulatus* genome. It should be noted that no specific transcripts have been identified as products of the various *bch* genes, and until recently crt genes (Giuliano et al., 1988). All the following data have been derived from experiments using probes that encode for more than one gene which are not necessarily co-transcribed. Initially, in Rb. capsulatus Clark et al., (1984) showed that a decrease in oxygen tension resulted in a 3- fold increase in transcript level of a number of bch genes but reported no increase in the transcript level for crt genes. Biel and Marrs (1985) also showed that carotenoid gene expression was not directly regulated by oxygen. However, Scolnik and co-workers recently monitored the levels of transcripts using probes specific for individual crt genes and showed that a 2 - fold increase in carotenoid content of Rb. capsulatus cells on a shift from aerobic to anaerobic conditions could be accounted for by an increase in the levels of transcripts encoding crt genes (Giuliano et al., 1988).

Zhu and co-workers showed that the level of mRNA encoding *bch* genes in *Rb. capsulatus* decreased on a shift from anaerobic to aerobic growth conditions (Zhu *et al.*, 1986a). However, conclusions deduced from measurements of transcript levels in steady state growth conditions are contradictory. The levels of transcripts specific for *crt* and *bch* genes were higher in high oxygen steady state conditions when compared to low oxygen conditions (Zhu and Hearst, 1986). Conversely, the level of transcripts encoding *bch* genes was higher in low light conditions, whereas the level of transcripts encoding *crt* genes was lower (Zhu and Hearst, 1986). It is clear that more work is required to sort out the expression of *bch* and *crt* genes in response to light and oxygen using gene specific probes, Northern analysis and transcriptional fusions.

Recently, Scolnik and co-workers produced a detailed map of the carotenoid gene cluster of *Rb. capsulatus* and concluded from nuclease Sl protection analysis that all the *crt* genes are organised as single transcriptional units with the exception of *crt*I/B, and that *crt*A, *crt*C and *crt*E transcript levels were elevated in anaerobic conditions. Now that transcripts have been linked with *crt* genes an increase understanding of how the levels of *crt* transcripts are affected by light and oxygen should be forthcoming.

1.7.5. Oxygen regulated promoters

From the studies mentioned previously, it is clear that oxygen tension plays a significant role in the expression of genes for photosynthetic membrane assembly. However, only one oxygen regulated promoter has been identified. Bauer and Marrs (1988) mapped two promoters -699 and -511 bp upstream of the start of *puf*B using interposon insertions and S1 mapping. Transcription of the -699 promoter was found to be regulated by oxygen in a manner comparable to that found for *puf* transcripts. The -511 promoter was responsible for low level constitutive expression. Similar promoters which show a high degree of homology to those of *Rb. capsulatus* have been found in comparable places in the *puf* operon of *Rb. sphaeroides* (Ashby, 1988).

Kranz and Haselkorn (1986) have shown, using lac fusions, that the anaerobic expression of the *nif* operon of *Rb. capsulatus* is controlled by the supercoiling of DNA. Recently, Zhu and Hearst (1988) showed that the presence of gyrase inhibitors novobiocin and coumermycin reduced the levels of mRNA transcripts encoding for genes involved in bchl biosynthesis and LH and RC complexes. It seems possible that the level of supercoiling of DNA, in response to the levels of oxygen, has an effect on the differential expression of these genes in *Rb. capsulatus*.

1.8. Important aspects of control of membrane assembly

It is clear from studies of mRNA transcript levels that photosynthetic membrane assembly is affected by transcription rates, transcript stability and post-transcriptional modification. However, the assembly of photosynthetic membranes is affected by other parameters which need to be considered. The photosynthetic apparatus of *Rb. sphaeroides* is located on distinct intracytoplasmic membrane (ICM) invaginations. On mechanical disruption of cells ICM form spherical, closed, photosynthetically competent vesicles called chromatophores (Niederman and Gibson, 1978). When chromatophores are prepared from cells undergoing a switch from high to low oxygen concentration, which initiates photosynthetic membrane formation, a pigmented band can be separated from ICM on the basis of its relative low mobility on rate-zone sucrose gradients. This fraction contains RC, LHI and LHII complexes. Pulse chase studies have suggested that this upper pigmented fraction is enriched in newly synthesised light harvesting and reaction centre complexes (Neiderman *et al.*, 1979,1981). This together with measurements of the sequence in which pigment-protein complexes appear in the membrane (Hunter *et al.*, 1982; Reilly and Niederman, 1986), fluorescence emission (Hunter *et al.*, 1979a) and photochemical studies (Hunter *et al.*, 1979b), suggest that there are specific sites for membrane development. Intracytoplasmic membrane development starts with the formation of

LHI-reaction centre 'cores' at sites of limited invagination of the cytoplasmic membrane which give rise to the slowly sedimenting pigmented band in sucrose gradients. This is followed by the addition of LHII units (Hunter *et al.*, 1982, Klug *et al.*, 1985, Rielly and Niederman, 1986), a process which is reflected in the levels of mRNA transcripts encoding the polypeptides of photosynthetic complexes (Hunter *et al.*,1987; Klug *et al.*, 1985). Mutants lacking LHII are unable to synthesise mature spherical intracytoplasmic membranes, forming instead long elongated tubes (Hunter *et al.*, 1988b, Kiley *et al.*, 1988). It has been suggested that the curvature of photosynthetic membranes may be imposed by aggregates of LHII complexes (Neil Hunter, personal communication).

1.8.1. The role of carotenoids in membrane assembly

Carotenoidless (blue-green) mutants of *Rhodospirillaceae* grow photosynthetically although they are more sensitive to photooxidative damage when conditions of high light intensity are combined with high oxygen concentrations. Carotenoidless mutants of both *Rb. capsulatus* and *Rb. sphaeroides* possess RC and LHI complexes but lack the absorbance spectrum attributed to LHII (B800-850) (Marrs,1978a). In *Rb. capsulatus* a third polypeptide of 14 kD which does not bind bchl or carotenoid, is found in isolated LHII complexes (Shiozawa *et al.*, 1982). Some carotenoidless strains of *Rb. capsulatus* have been found that lack the 14 kD polypeptide (Youvan *et al.*, 1982; Zsebo and Hearst, 1984; Kaufman *et al.*, 1984). Although an equivalent third LHII polypeptide has not yet been found in *Rb. sphaeroides*, R26, a blue green mutant widely used for reaction centre and LHI isolation, also lacks the LHII absorption peaks. A derivative of R26 called R26.1 was found to have regained the LHII complex, however, only the B850 absorbency peak was present (Davidson and Cogdell, 1981). Marrs (1978a) pointed out that when carotenoidless mutants regained the ability to synthesise carotenoids an altered LHII absorption spectra was still observed. This could be explained if the loss of carotenoid biosynthesis leads to a secondary mutation which reduced the effectiveness of the LHII complex, perhaps reducing the degree of photooxidative damage (Marrs, 1978a). It is clear that the loss of carotenoids has a profound effect on the LHII complex.

1.8.2. The role of bacteriochlorophyll in membrane assembly

Although it has been known for a long time that mutants with deficiencies in bacteriochlorophyll biosynthesis cannot photosynthesise (Lascelles, 1978). It was only with the advent of RC, LHI and LHII isolation and subsequent use of immunological methods coupled with pulse-chase experiments that the role of bchl in

photosynthetic membrane assembly has been investigated. Dierstein showed that inhibition of bchl synthesis at the level of 5-aminolevulinate dehydratase resulted in an immediate halt in the formation of pigment-protein complexes (Dierstein and Drews, 1982). Using a bacteriochlorophyll-less mutant of *Rb. capsulatus* it was demonstrated immunochemically that LHI, LHII and RC polypeptides were not accumulated in membranes, but underwent rapid degradation. This suggested that bchl is needed to stabilize pigment-protein complexes in the membrane (Dierstein, 1983; Dierstein *et al.*, 1984; Klug *et al.*, 1985). It has been noted that the RC-H polypeptide, which does not bind bchl, appears stable and is immunochemically detectable in *Rb. sphaeroides* cells with no bacteriochlorophyll (Donohue *et al.*, 1986a). Klug *et al.*, (1986) showed that inhibition of bchl synthesis in *Rb. capsulatus* did not impair the formation of mRNA for pigment-binding polypeptides, however the bchl-less RC and LH complexes were unstable. In bacteriochlorophyll-less mutants of *Rb. capsulatus*, the levels of *puf* and *puc* transcripts were uncoupled from oxygen control, showing that bchl itself must play an important role in oxygen regulation (Klug *et al.*, 1986).

The discovery that the *puf*Q gene product is essential for bchl synthesis may provide the answers to how photosynthetic membrane development is regulated. Bauer has pointed out that the *puf*Q gene product exhibits a high degree of homology with the quinone binding pocket of RC-L and RC-M, suggesting that the *puf*Q gene product may interact with the quinone pool (Bauer and Marrs, 1988). Cohen-Bazire *et al.*,(1957) originally suggested that a bchl regulatory protein could be monitoring the electron transport systems involved in photosynthetic and respiratory electron transport in which quinones play an essential role. Further, it has been observed that Mg insertion into protoporhyrin XI is inhibited by the addition of quinone analogs and uncouplers of electron transport (Gorchein, 1973) and that a membrane bound protein of the same size as the *puf*Q gene product binds azido-atrazine, a competitive inhibitor of quinone (Brown *et al.*, 1984). Although it has still to be proved that the *puf*Q gene product is a quinone binding protein, it adds an exciting new dimension to regulation of photosynthetic membrane assembly.

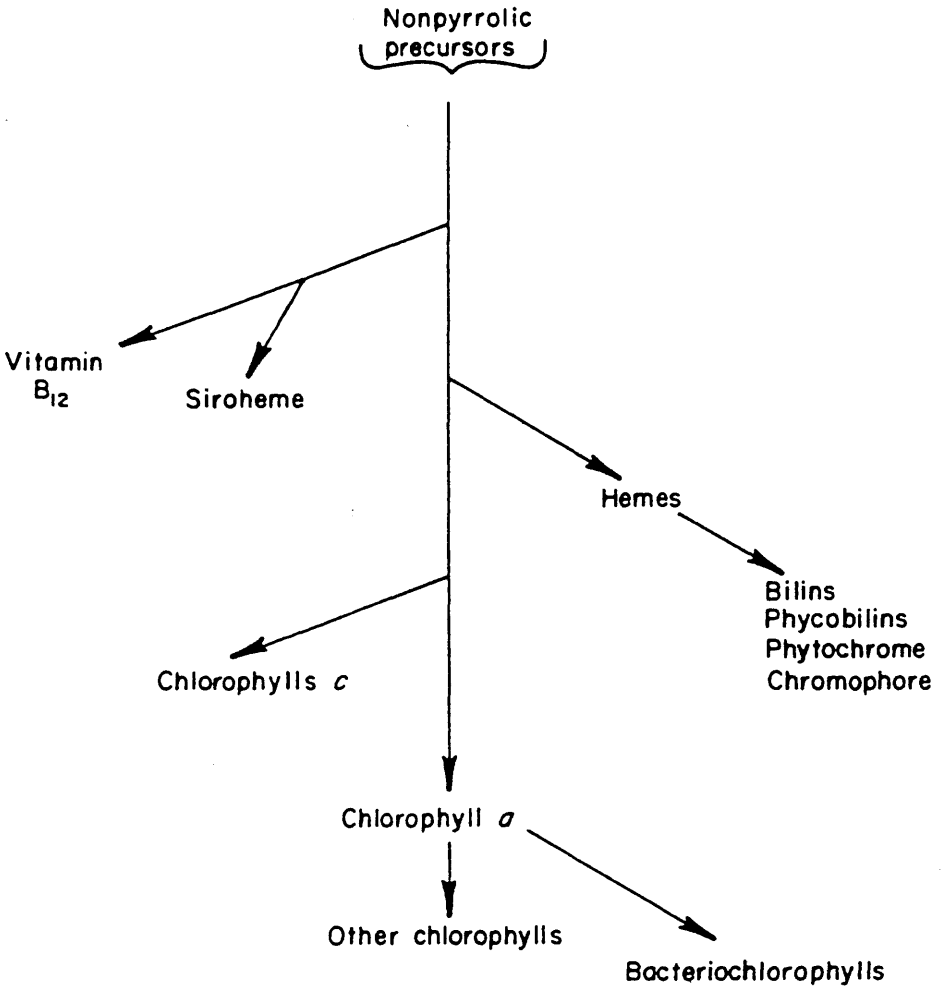


Figure 1.1

An outline of the tetrapyrrole biosynthetic pathway showing its major products (from Castlefranco and Beale, 1981).

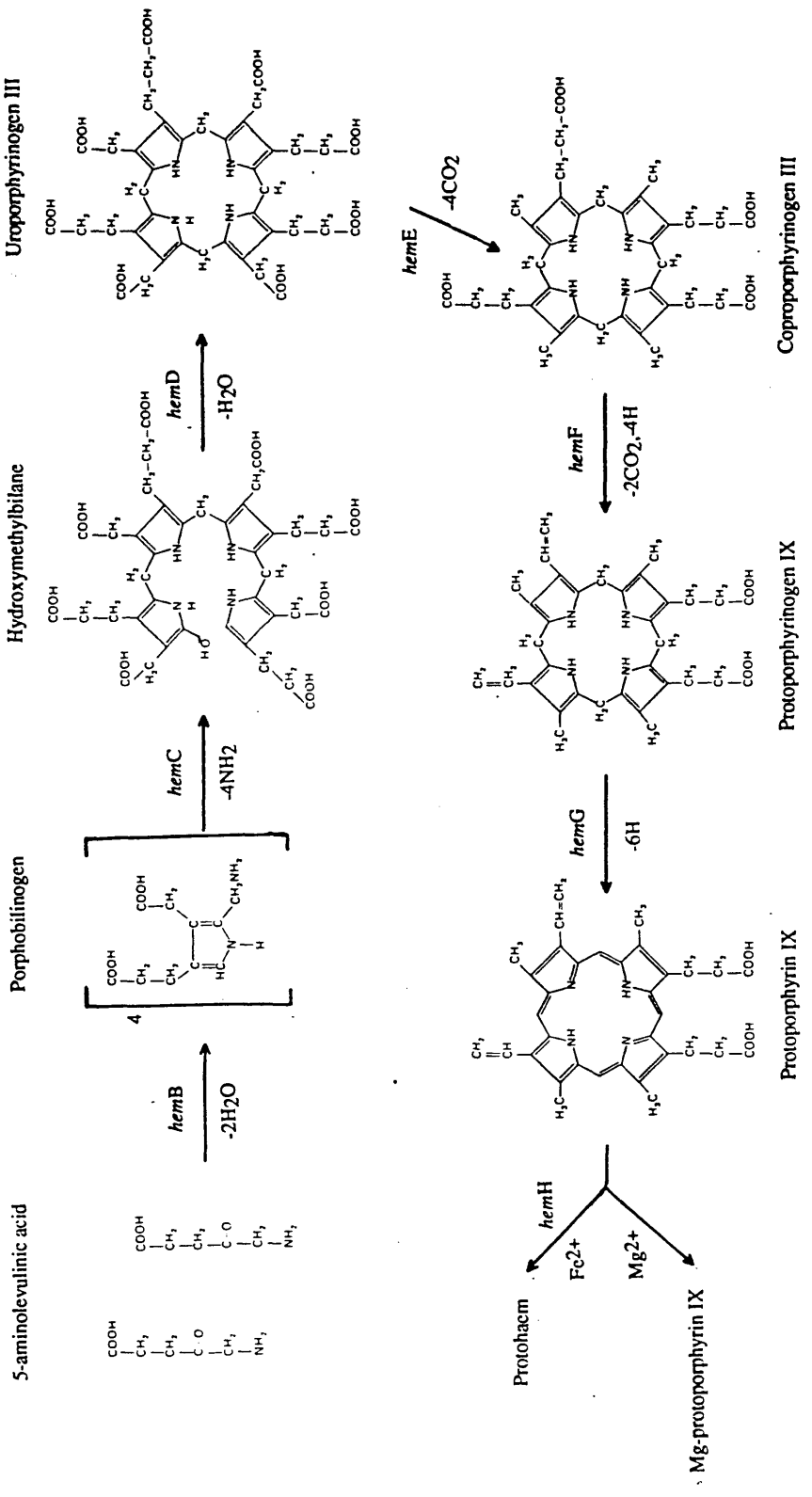


Figure 1.2A

The common tetrapyrrole pathway from 5-aminolevulinic acid to the metal chelation step at the branch point between haem and bacteriochlorophyll biosynthesis. The structures of each isolated intermediate along with the genes involved at each stage are shown.

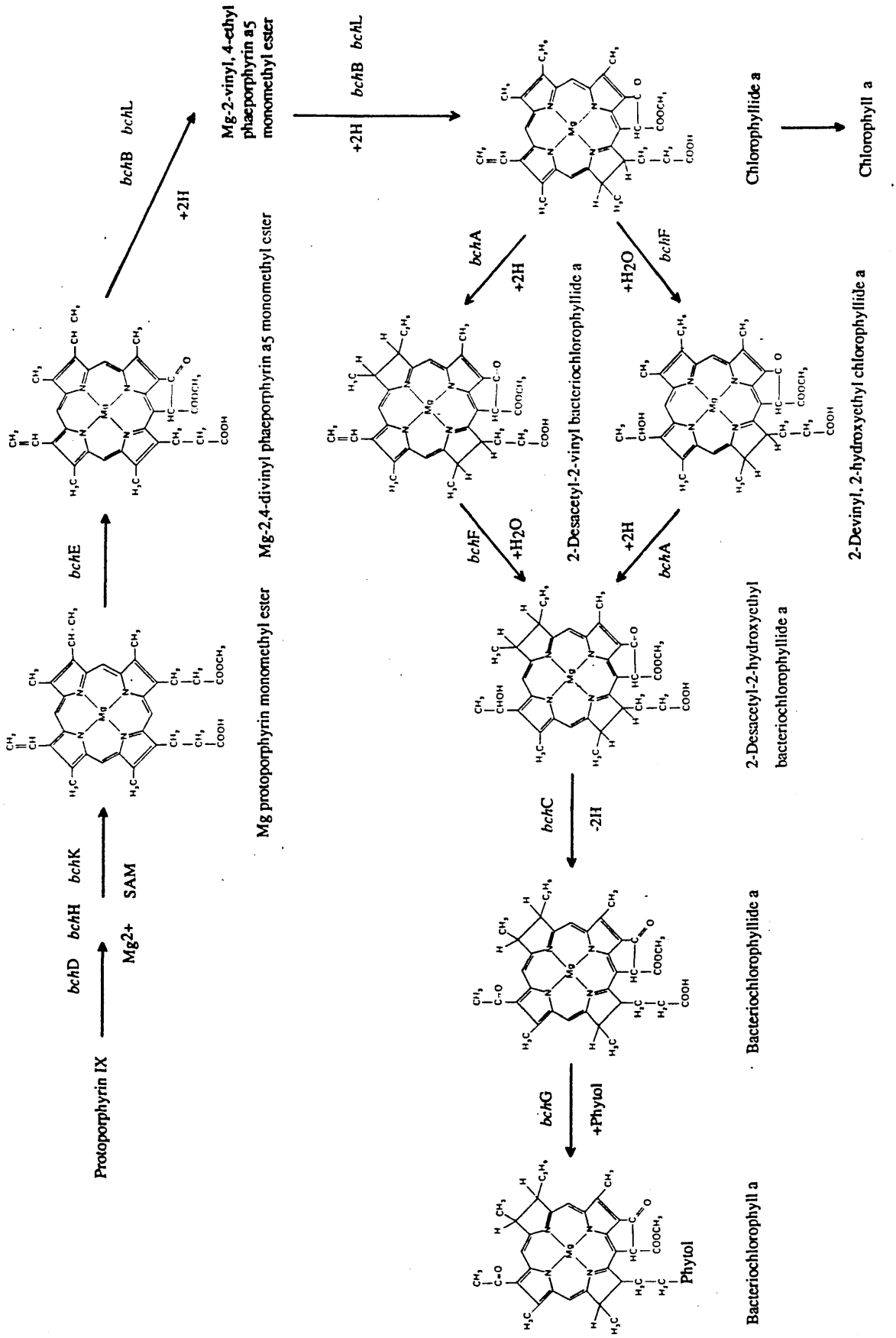
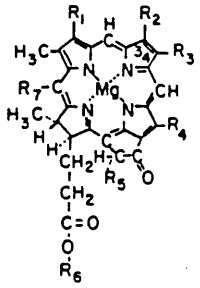


Figure 1.2B

The bacteriochlorophyll biosynthesis pathway from the point of Mg^{2+} insertion into protoporphyrin IX. The structures of the isolated intermediates are shown along with the genes involved at each stage (based on Biel and Marrs, 1983). The point at which chlorophyll would be synthesized in algae and plants is also shown.



Pigment	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Chlorophyll *a*[a]	$-CH{=}CH_3$	$-CH_3$	$-CH_2{-}CH_3$	$-CH_3$	$-C(=O)-O-CH_3$	P	$-H$
Chlorophyll *b*[a]	$-CH{=}CH_3$	$-C(=O)-H$	$-CH_2{-}CH_3$	$-CH_3$	$-C(=O)-O-CH_3$	P	$-H$
Bacteriochlorophyll *a*	$-C(=O)-CH_3$	$-CH_3$[b]	$-CH_2{-}CH_3$[b]	$-CH_3$	$-C(=O)-O-CH_3$	P/Gg	$-H$
Bacteriochlorophyll *b*	$-C(=O)-CH_3$	$-CH_3$[c]	$=C(H)-CH_3$[c]	$-CH_3$	$-C(=O)-O-CH_3$	P	$-H$
Bacteriochlorophyll *c*	$-CH(OH)-CH_3$	$-CH_3$	$-C_2H_5$ / $-C_3H_7$ / $i-C_4H_9$	$-C_2H_5$ / $-CH_3$(?)	$-H$	F	$-CH_3$
Bacteriochlorophyll *d*	$-CH(OH)-CH_3$	$-CH_3$	$-C_2H_5$ / $-C_3H_7$ / $i-C_4H_9$	$-C_2H_5$ / $-CH_3$(?)	$-H$	F	$-H$
Bacteriochlorophyll *e*	$-CH(OH)-CH_3$	$-C(=O)-H$	$-C_2H_5$ / $-C_3H_7$ / $i-C_4H_9$	$-C_2H_5$	$-H$	F	$-CH_3$

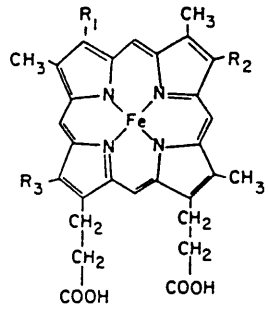
P = phytyl: $-CH_2CH{=}C(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-(CH_2)_3-CH(CH_3)-CH_3$

F = farnesyl: $-CH_2-CH{=}C(CH_3)-(CH_2)_2-CH{=}C(CH_3)-(CH_2)_2-CH{=}C(CH_3)-CH_3$

[a]More specifically, 2-vinyl-4-ethyl-Chl. [b]No double bond between C-3 and C-4; additional H-atoms at positions C-3 and C-4. [c]No double bond between C-3 and C-4; an additional H-atom in position C-3.

Figure 1.3A

The structure of common chlorophyll and bacteriochlorophyll molecules (from Gloe *et al.*, 1975).



(a) $R_1, R_2 = -CH{=}CH_2$; $R_3 = -CH_3$; = PROTOHEME

(b) $R_1 = -CH(OH)-CH_2-CH_2-CH{=}C(CH_3)-CH_2-CH_2-CH{=}C(CH_3)-CH_2-CH_2-CH{=}C(CH_3)-CH_3$

$R_2 = -CH{=}CH_2$; $R_3 = -CHO$; = HEME *a*

(c) $R_1, R_2 = -CH(S\text{-PROTEIN})-CH_3$; $R_3 = -CH_3$; = HEME *c*

Figure 1.3B

Haems found in photosynthetic bacteria (from Rebeiz and Lascelles, 1982).

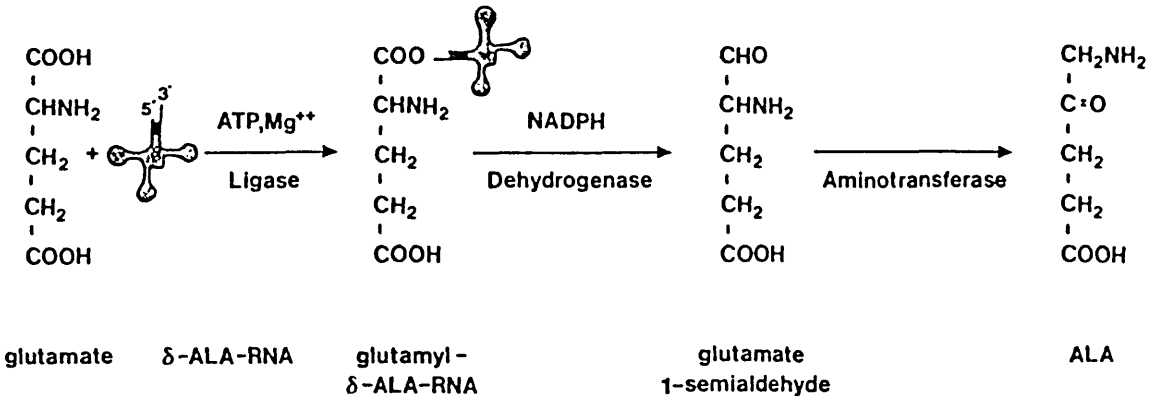


Figure 1.4B

The C_5 pathway showing the enzymes and intermediates involved in the formation of 5-aminolevulinic acid from glutamate (from Kannangara *et al.*, 1988).

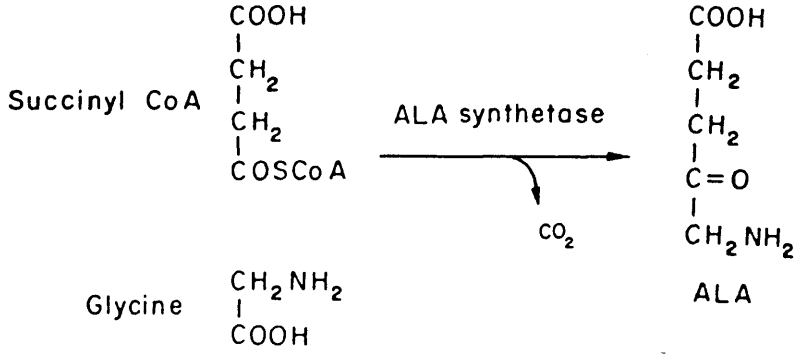


Figure 1.4A

The Shemin pathway showing the formation of 5-aminolevulinic acid from the condensation of glycine and succinyl CoA.

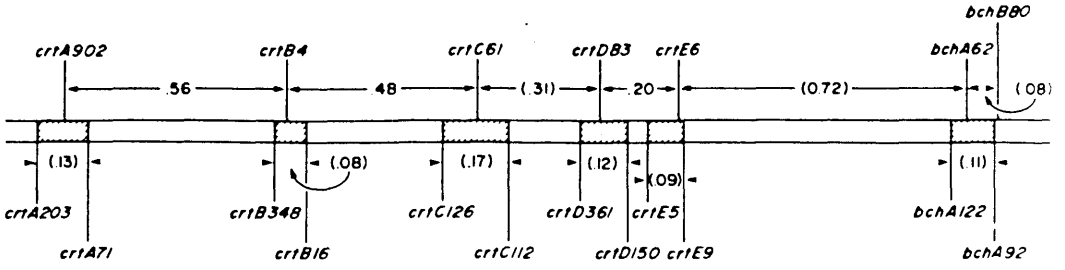


Figure 1.5A (Yen and Marrs, 1976)

The first genetic map of the region for carotenoid and bacteriochlorophyll biosynthesis in *Rb. capsulatus* produced using the gene tranfer agent (GTA). The numbers on the map represent, in map units, the distances between genes and show the minimum length of each gene.

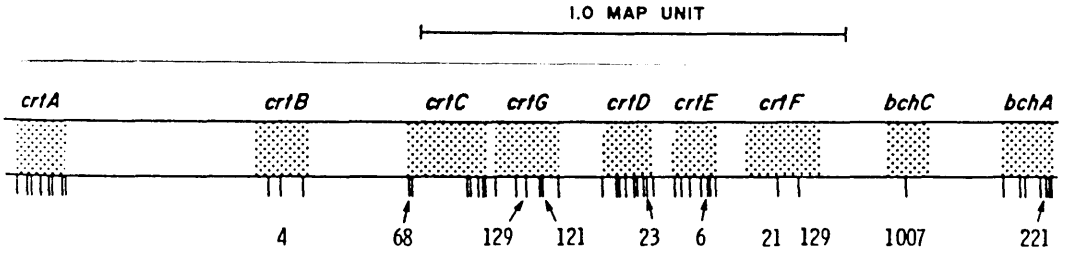


Figure 1.5B (Scolnik *et al.*, 1980)

The genetic map of the photopigment region of *Rb. capsulatus*. Each dash represents a single mutation and the distances involved were calculated from cotransfer experiments using the GTA system.

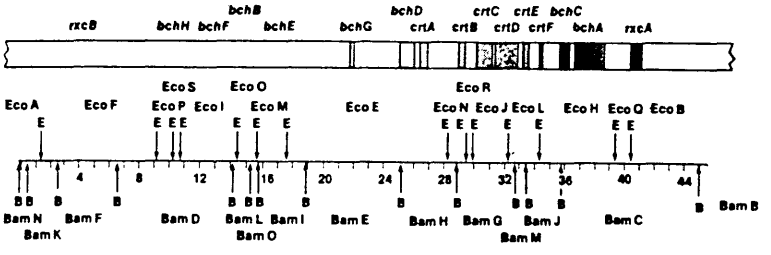


Figure 1.5C (Taylor *et al.*, 1983; Biel and Marrs, 1983))

Alignment of the genetic and restriction maps of the region of the *Rb. capsulatus* chromosome carrying genes involved in the photosynthetic apparatus. The 46 kb of *Rb. capsulatus* DNA carried on the R-prime plasmid pRPS404 is shown. The shaded region indicates genetically determined map positions of mutations bearing the same phenotype. The positions of the genes for which no shaded area is indicated were determined using a conjugation mediated marker rescue technique.

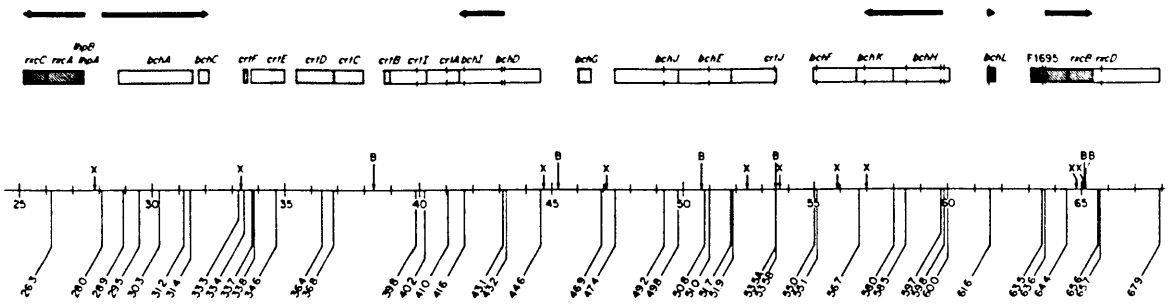


Figure 1.5D (Zsebo and Hearst, 1984)

Genetic-physical map of the photosynthetic gene cluster of *Rb. capsulatus* using Tn5.7 mutagenesis. The vertical ticks refer to transposon insertions. The boundaries for each loci, indicated by vertical lines were determined by DNA sequencing (dark shading), data previously published obtained from gene transfer agent crosses (light shading) or Tn5.7 mutagenesis and complementation analysis (no shading). The arrows above postulate direction and extent of transcription for some photosynthesis genes.

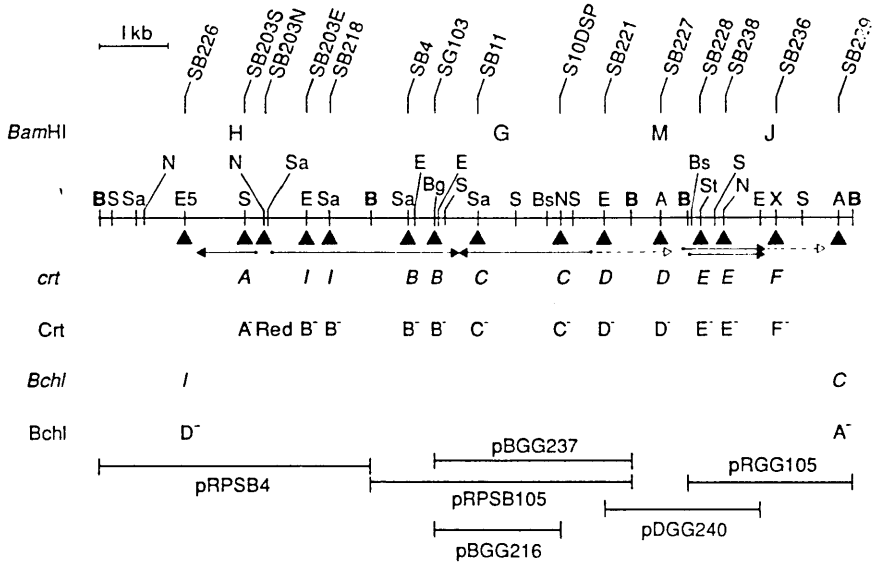


Figure 1.5E (Giuliano *et al.*, 1988)

Genetic-physical map of the *Rb. capsulatus* carotenoid cluster. Triangles represent the positions of each interposon insertion. *crt* and *Bchl* refer to the genotypes, whereas Crt and Bchl refer to the phenotypes produced by each insertion. Restriction endonuclease sites: A, *Apa* I; B, *Bam* HI; BG, *Bgl* II; Bs, *Bst* EII; E, *Eco* RI; ES, *Eco* RV; N, *Nru* I; S, *Sph* I; Sa, *Sal* I; St, *Stu* I; X, *Xho* I. Solid arrows indicate direction of transcription. Dashed arrows show direction of transcription deduced from preliminary DNA sequence information.

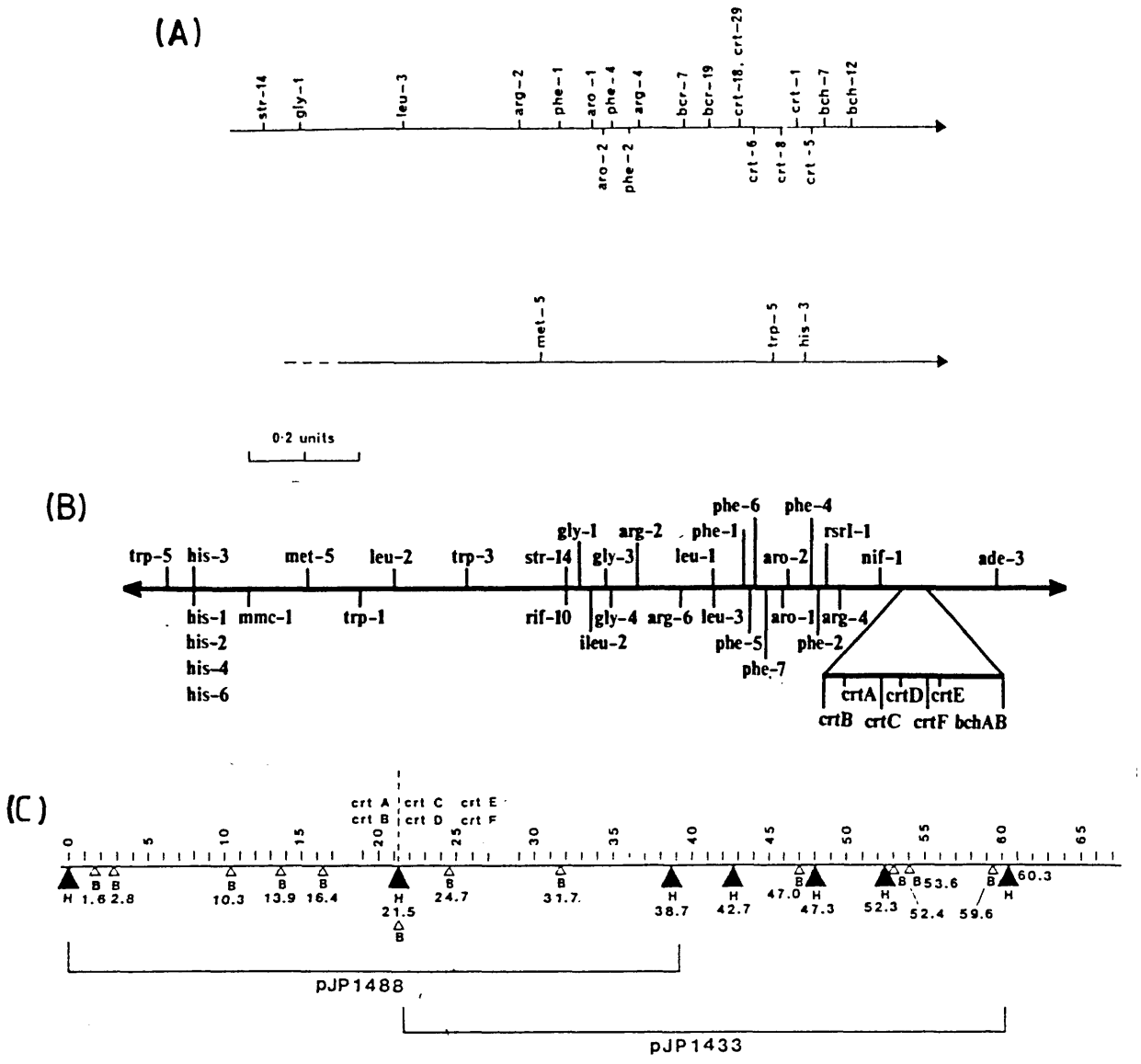


Figure 1.6 Genetic maps of *Rb. sphaeroides* showing genes involved in photosynthesis

(A) (Pemberton and Bowen, 1981)

Genetic linkage map of the *Rb. sphaeroides* chromosome determined from RP1::Tn501 mediated chromosome transfer. Crt indicates a carotenoid mutant and bch a bacteriochlorophyll mutant. However no phenotype descriptions were presented.

(B) (Bowen and Pemberton, 1985)

Linkage map of the *Rb. sphaeroides* chromosome. The relative positions of markers were determined using RP1::Tn501 and pR751::Tn813 promoted chromosome transfer. The photopigment gene cluster was drawn from data presented in Pemberton and Bowen (1981)(figure 1.6A). It is not explained why bch or crt mutants have been renamed or describes their phenotypes.

(C) (Pemberton and Bowen, 1986)

Physical and genetic map of cosmids bearing *Rb. sphaeroides* carotenoid genes. B = *Bam* HI, H = *Hind* III.

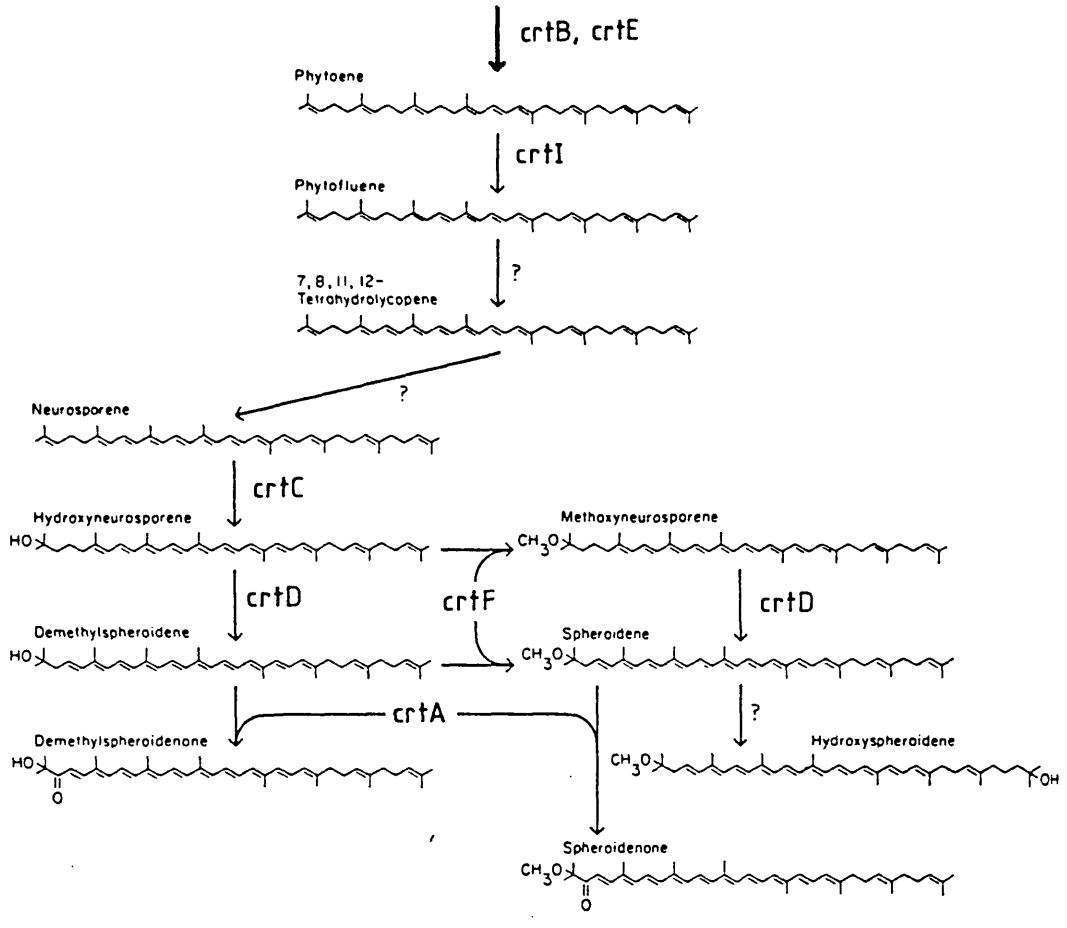


Figure 1.7

The proposed pathway for carotenoid biosynthesis in *Rb. capsulatus*. The steps affected by various mutations in *crt* genes are indicated (from Scolnik *et al.*,1980; Guiliano *et al.*, 1986).

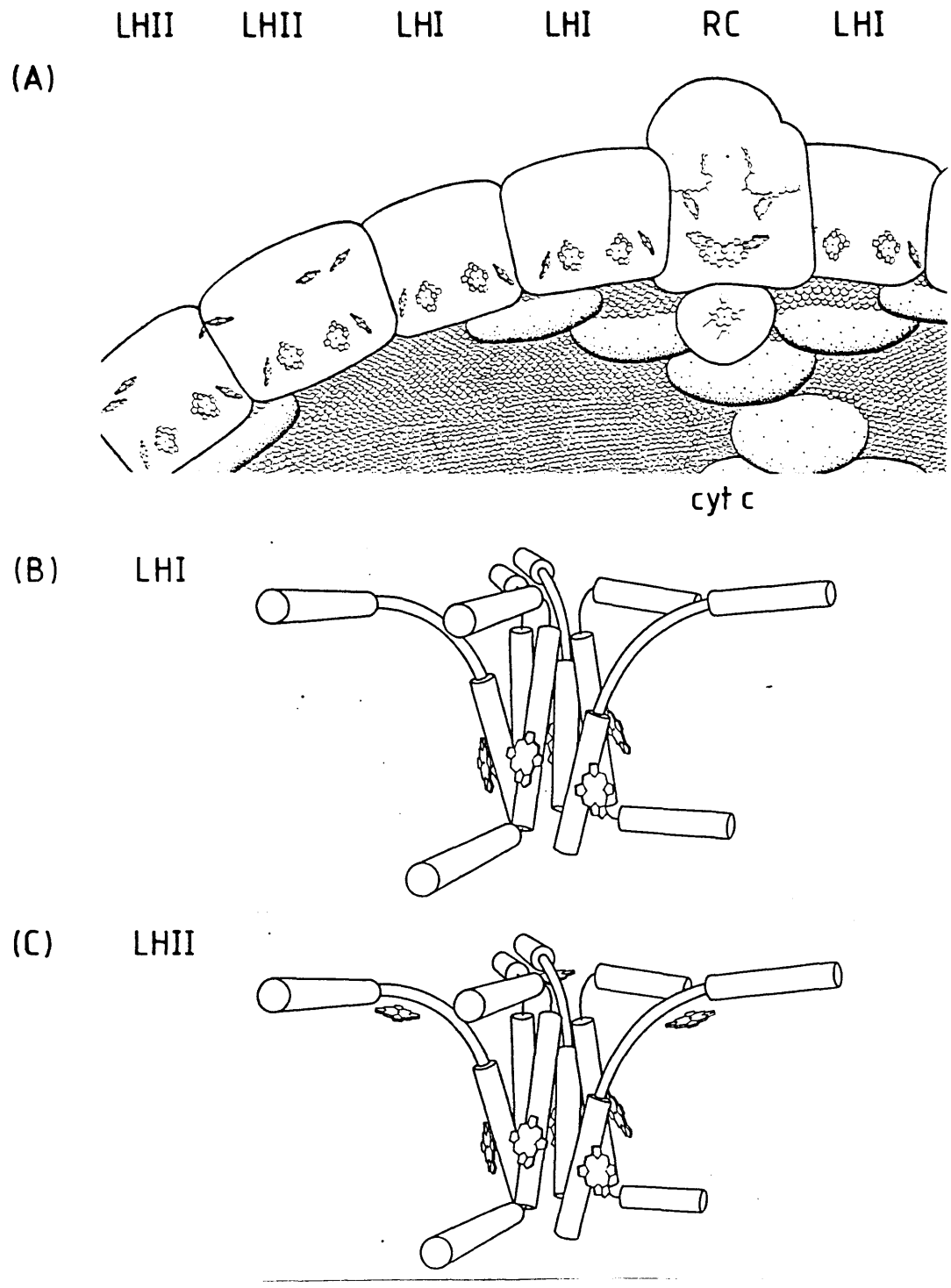


Figure 1.8 Models of *Rb. sphaeroides* pigment-protein complexes (drawn by J. Olsen)

(A) A model showing the arrangement of bacteriochlorophyll and proteins within the intracytoplasmic membrane and the close association of the extrinsic cytochrome c molecule on the periplasmic side of the membrane complex.

(B) A model of a LHI complex showing a possible minimal arrangement of 3∝ and 3ß polypeptides each binding a single bchl molecule. ∝helical regions are shown as cylinders. The six carotenoid molecules which are associated with the complex are not shown.

(C) A model of the LHII complex showing a possible minimal arrangement of 3∝ polypeptides each binding a single bchl, and 3ß polypeptides each binding two bchl molecules. The six carotenoid molecules are not shown.

CHAPTER 2

Materials and methods

2.1. Standard buffers, reagents and media.

Unless otherwise stated, the preparation and composition of all the buffers, reagents and media used in the work for this thesis are as outlined in Maniatis *et al.*, (1982). Culture media was made up with 'reverse osmosis' water obtained using the Milli-RO 15 water purification system from Millipore. The majority of inorganic chemicals and organic solvents were Analar grade reagents and were purchased from BDH Chemicals Ltd. All buffers and solutions used in the DNA and RNA work were prepared using deionized water obtained from the passage of reverse osmosis water through the nanopure deionisation system from Barnstead. Culture media and solutions used for DNA and RNA work were sterilized by autoclaving at 15 p.s.i. for at least 20 minutes, or by filtration through O.2μm disposable Scheider and Schnell Filters or O.2μm Nalgene sterilization filter units. Thermolabile substances such as antibiotics and vitamins were not added until autoclaved solutions had cooled below 55°C.

2.2. *E. coli* strains and plasmids.

Table 2.1 lists those *E. coli* strains described in this thesis, together with their genotype. For short term storage, *E. coli* strains were maintained on suitable selective plates at 4°C. For long term storage cultures were resuspended in 20% (w/v) glycerol/LB and stored at -80°C. Antibiotics were added at the following concentrations (μg/ml): kanamycin 30, neomycin 30, tetracycline 10, streptomycin 25, ampicillin 50, spectinomycin 50, chloramphenicol 25. Table 2.2 gives a list of plasmids used in this work.

2.3. *Rb. sphaeroides.*

Rb. sphaeroides strain 8253 was used throughout this work. For short term storage *Rb. sphaeroides* strains were maintained on M22+ Casamino acid plates at room temperature. For long term storage cultures were resuspended in 20% (w/v) glycerol/LB and stored at -80°C.

2.3.1. *Rb. sphaeroides* media.

Rb. sphaeroides was routinely grown on M22+ Casamino acids, an enriched version of M22 (Sistrom,1977). Table 2.3 lists the ingredients of M22+, solution C and 1000x vitamins stock solution. M22+ contained all M22+ ingredients plus vitamins. M22+ CAA contained all M22+ ingredients, plus vitamins and O.1% Casamino acids. M22+ was used for plating out matings between *E. coli* and *Rb. sphaeroides* to cut down background *E. coli* growth. Antibiotics were added to the following concentrations (μg/ml): kanamycin 20, neomycin 20, streptomycin 30, carbenicillin 1.

2.3.2. Low aeration growth of *Rb. sphaeroides* for maximum pigment production.

Rb. sphaeroides mutants were grown for spectral examination in the following way. Ten individual week old colonies were taken and placed in 10 mls of M22+ CAA in a 30 ml universal bottle (with suitable antibiotics). The culture was shaken at 180 rpm in a Gallenkamp orbital shaker at 32°C for 48 hours. The 10 ml culture was then tipped into a 100 ml conical flask containing 70 mls of M22+ CAA and grown for a further 48 hours. The culture was harvested and the cells and supernatant subjected to spectral examination (see section 2.7).

2.3.3. Growth of *Rb. sphaeroides* cultures undergoing low aeration induction of pigment synthesis.

The method of Niederman *et al.*, (1976) was used to induce bacteriochlorophyll synthesis in suspensions of *Rb. sphaeroides* under conditions of low aeration. Initially a 10 ml *Rb. sphaeroides* culture was grown up over 2 days. An absorbance reading at A680 was taken of the starter culture, and assuming a doubling time of 2 hours, a volume of the culture was added to 200 mls of M22+ CAA in a 2 litre flask, that would give a final A680 of around O.5 after overnight growth. These aerobic *Rb. sphaeroides* cultures were shaken at 200 rpm overnight at 32°C. When the nonpigmented *Rb. sphaeroides* cultures had reached an absorbance of 0.5 at 680 nm, they were quickly harvested and resuspended to a final A680 = 2 in 80 mls of M22+ CAA. The resulting semi-aerobic cultures were then shaken at 200 rpm at 32°C in 100 ml conical flasks. Cells were harvested and used for spectral analysis, pigment extraction and RNA extraction typically at 0, 0.5, 1, 2, 4 and 6 hours after start of pigment induction.

2.3.4. Growth of *Rb. sphaeroides* in anaerobic conditions in the light.

To create lighting conditions of 30 W/m^2 an array of 40W light bulbs were placed at distances around 40 cm from anaerobic jars/bottles. The air was circulated using an electric fan to ensure that the immediate air temperature did not rise above 32°C. Anaerobic conditions in liquid cultures was achieved by placing *Rb. sphaeroides* in glass bottles completely filled with M22+ CAA, then tightly stoppered. The BBL Gaspak system (Becton Dickinson) which utilizes disposable hydrogen and carbon dioxide generator envelopes was routinely used for anaerobic growth of *Rb. sphaeroides* on agar plates.

2.4. Nucleic acid manipulation.

2.4.1 Plasmid DNA preparation.

A modification of the Holmes and Quigley (1981) technique was used extensively throughout this work. It performed particularly well with large plasmids, providing plasmid DNA largely free of genomic DNA. For small scale preparation of plasmid DNA a 10 ml overnight culture of *E. coli* was used. The cells were harvested and resuspended in 250µl of 25%(w/v)sucrose/50mM Tris-HCl pH8 in a 1.5 ml eppendorf. Then 40µl of 10mg/ml lysozyme was added, the tube shaken and left at room temperature for 2 minutes. 125µl of O.2M EDTA pH8 was added quickly followed by 250µl of 2% Trition X-100/50mM Tris-HCl pH8. The tube was shaken and centrifuged at 12,000 rpm in a bench Microfuge. The supernatant was extracted twice with an equal volume of phenol/chloroform (1:1 v/v) before isopropanol precipitation of nucleic acids. The nucleic acid pellet was washed in 80% EtOH desiccated and resuspended in 400µl TE. A further phenol/chloroform (1:1 v/v) extraction was followed by a chloroform extraction before ethanol precipitation of nucleic acids. The nucleic acid pellet was routinely dissolved in 20µl of TE and stored frozen at -20°C. Depending on the vector used the following volumes of each plasmid preparation was subjected to analysis by restriction endonuclease digestion: pNH2-10µl, pSUP202-5µl, pUC18/19-O.5µl. These variations can be accounted for because of differences in plasmid copy number per cell.

For large scale preparations of plasmid DNA a 1 litre overnight culture of *E. coli* cells was grown and harvested. The cells were resuspended in 50 mls of 50mM Tris-HCl pH8/25%(w/v) sucrose in a 250 ml centrifuge bottle. 8 mls of 10mg/ml lysozyme in 50mM Tris-HCl pH8 was added, the bottle shaken and left at 37°C for 20 minutes. 25 mls of O.2M EDTA pH8 and 50 mls of 2% Trition x-100/50mM Tris-HCl pH8 were added in quick succession and mixed in gently. The genomic DNA

and cell debris were pelleted by centrifugation at 12,000 rpm in a Beckman JA-14 rotor for 20 minutes. The supernatant was extracted once with phenol/chloroform (1:1 v/v) and the nucleic acid precipitated using isopropanol. After centrifugation the resulting pellet was washed with 80% ethanol, desiccated and resuspended in 9 mls of TE. These nucleic acids were fractionated on a caesium chloride density gradient as described in Maniatis *et al.*, (1982). The plasmid band was visualised using a uv light source, removed and the ethidium bromide extracted several times with caesium chloride saturated isopropanol. The plasmid DNA was dialysed against several changes of TE. Plasmid DNA was routinely stored frozen at -20°C.

2.4.2. RNA preparation from *Rb. sphaeroides*.

A method of extracting RNA from E. coli (Dr M.J.McPherson, personal communication) was adapted for use with *Rb. sphaeroides*. Special care was taken to ensure that all solutions and equipment were freshly autoclaved, and gloves were worn throughout the isolation procedure. An equal volume of ice cold extraction buffer (80mM Tris-HCl pH7.5, 10mM $MgCl_2$, 25mM NaN_3, 10mM ß-mercaptoethanol, 200µg/ml chloramphenicol) was added to the *Rb. sphaeroides* culture. The cells were quickly spun down in a Beckman JA-14 rotor at 8,000 rpm for 5 minutes.They were quickly resuspended in 1 ml of TE and placed in a 50 ml polyurethane centrifuge tube. 4 mls of boiling 0.5% SDS in TE was added and the tube placed in boiling water for 5 minutes. Then 100µl of 2M KCl was added and the tube cooled on ice for 5 minutes. The cell debris was pelleted at 12,000 rpm in a Beckman JA-20 rotor for 10 minutes. The supernatant was immediately mixed with 0.5g CsCl per ml, and ß-mercaptoethanol added to 170mM. This mixture was layered onto a 2 ml cushion of 5.7M CsCl, 10mM Tris-HCl, 100mM EDTA at pH8, in a 10 ml ultra-centrifuge tube. The tubes were spun at 35K in a kontron TST41.14 rotor for 18 hours. The liquid was carefully removed and the RNA pellet quickly dissolved in 2 mls of TE. 5 mls of ethanol was then added; RNA was routinely stored as an ethanol precipitate at -20°C.The RNA concentration was measured as described in Maniatis *et al.*, (1982).

2.4.3. Preparation of *E. coli* and *Rb. sphaeroides* genomic DNA.

80 mls of an overnight *E. coli* culture or a 2 day old *Rb. sphaeroides* culture was harvested and resuspended in 8 mls of TE in a universal bottle. 1 ml of 10mg/ml lysozyme in TE was added, shaken,and the mixture incubated at 37°C. for 10 minutes. The genomic DNA was fractionated on a caesium chloride density gradient as described in Maniatis *et al.*,(1982). The genomic band was identified and treated as in section 2.4.1 except that genomic DNA was routinely stored at 4°C.

2.4.4. Preparation of competent *E. coli* cells

Highly efficient competent cells were routinely prepared using 'Protocol 3' as described by Hanahan (1985). In this method cells undergoing log-phase growth are pelleted and incubated sequentially with two solutions termed RFl (100 mM RbCl, 50mM $MnCl_2$, 30mM potassium acetate, 10mM $CaCl_2$, 15% (w/v) glycerol pH 5.8) and RF2 (10mM MOPS pH 6.8, 10mM RbCl, 75mM $CaCl_2$, 15% (w/v) glycerol). The cells could be stored at -80°C in RF2. The fraction of competent cells produced by this method was usually of the order of 0.5 to 1%.

2.4.5. Transformation of *E. coli.*

Competent cells which were stored at -80°C were allowed to thaw on ice. To about l-5 µl of DNA (around 1 µg) in a 1.5 ml eppendorf tube, 50 µl of thawed competent cells was added. The tube was gently tapped to mix the contents and the cells allowed to incubate on ice for 30-45 minutes. The cells were 'heat shocked' at 42°C for 60-120 seconds then placed on ice for a further 10 to 20 minutes. 150 µl of SOB medium (Hanahan, 1985) was added and the tube placed at 37°C for 1 hour with occasional inversions. The bacterial suspension was then spread onto an appropriate selective agar plate and incubated at 37°C overnight.

2.4.6. Restriction enzyme digestions.

Digestion with retriction endonucleases (purchased from Northumbria Biologicals Ltd) were carried out in buffers according to manufactures instructions. For 'miniprep' plasmid DNA a typical digest contained 1 µg of DNA in 20 µl of lx salt buffer with 2-10 units of enzyme. 2 µl of 10xBSA/spermidine (2.5mg/ml BSA, 40mM spermidine) was routinely included to overcome problems with impurities. In addition, heat treated pancreatic RNAase was added to all digestions to a final concentration of 50 µg/ml to remove RNA. Digestions were commonly left overnight. For the purposes of mapping restriction sites, 20 µg of $CsCl_2$ purified plasmid DNA was digested in 20 µl total volume. The digest was divided between two 25cm gels, one loaded with 19 µl of digest and the other with the remaining
1 µl. This procedure ensured that bands smaller than 1 kb were clearly seen and that bands over 4 kb were not overloaded. Routinely, 1% agarose gels were prepared in lxTBE using ultrapure agarose purchased from Bethesda Research Laboratories. Both gel and lxTBE running buffer contained 0.5 µg/ml EtBr. Nucleic acid bands were visualised on a 254 nm uv light box and were sized by comparing their mobility to

visualised on a 254 nm uv light box and were sized by comparing their mobility to that of DNA fragments of known size, usually *Bgl* I and *Hind* III digestions of lambda DNA. Two photographic methods were used to record gels

1. Polaroid type 677 film in a CU-5 polaroid camera with an orange and a polarizing filter, fll and an exposure time of l0-20 seconds. Film was processed according to manufactures instructions.
2. Ilford pan F 135 film in a Practika MTL 5B camera with a polarizing and an orange filter, f5.6 and exposure time 30-120 seconds. The film was developed in full strength Promicol (May and Baker Ltd.) at 24°C for 9 minutes, inverting the tank every 30 seconds. The developer was discarded, and the film washed once in 2% acetic acid for 30 seconds, and once with tap water. Finally 1+4 Ilford Hypan fixer was added and left for 5 minutes with occasional agitation. The film was washed in clean running water for 30 minutes,and finally rinsed in reverse osmosis water containing a drop of 326 photographic wetting agent (Johnsons Chemicals).

2.4.7. Recovery of DNA from agarose.

Usually, the DNA fragment of interest to be cloned was purified using the dialysis bag method (Maniatis *et al.*,1982). After electrophoresis through a gel made from SeaKem GTG agarose purchased from FMC Bioproducts, the desired fragment was excised and placed in a dialysis bag overflowing with 1xTE. Most of the buffer was removed to leave a small volume around the gel slice without any air bubbles. The bag was then clamped at both ends using dialysis tubing clips (purchased from Gallenkamp), immersed in an electrophoresis tank containing 1 x TBE, a current passed (usually at 80 mA) until the DNA had migrated from the gel onto the wall of the bag. This process was monitored by viewing ethidium bromide fluorescence under long-wavelength uv. The current was reversed for about one minute to release the DNA from the wall into the buffer surrounding the gel slice. After opening the bag carefully, the buffer was removed and the gel slice washed in a small volume of lxTE. The washings were combined and extracted with phenol/chloroform (1:1 v/v) and finally chloroform, before being precipitated. The pellet was washed with 80% EtOH/H_2O, dried *in vacuo* and dissolved in TE ready for use. This solution was routinely examined by agarose gel electrophoresis to test for the purity of the fragment and its concentration.

2.4.8. Dephosphorylation of DNA.

When required, the 5' ends of DNA fragments were dephosphorylated using calf intestinal alkaline phosphatase, molecular biology grade, from BCL. This was frequently used to dephosphorylate cut vector DNA in order to increase the frequency of recombinant clones. Routinely, 1μl of enzyme (approximately 23U/μl) was added to the restriction digestion mixture after digestion had occurred, and the incubation continued for a further 30 minutes at 37°C. To inactivate the phosphatase, O.3 vol of EGTA (0.2M, pH 7.3) was added and the mixture heated at 65°C for 10 minutes. This was followed by phenol/chloroform (1:1 v/v) extraction and ethanol precipitation of the DNA.

2.4.9. DNA ligation reactions.

A typical ligation mixture consisted of 100 ng of vector, at least a molar equivalent of insert, 2μl of 5x ligation buffer (100mM Tris-HCl pH7.6, 100mM $MgCl_2$, 100mM DTT, 6mM ATP, 25% PEG 6000), and 1 unit of T4 DNA ligase in a total volume of 10μl. The reaction mixture was routinely left overnight at 16°C or at room temperature for 4 hours. Usually 5μl of this ligation mixture was used to transform competent cells.

2.4.10. Radiolabelling of DNA for probes.

DNA was routinely labelled using the method of Flinberg and Vogelstein (1983). To reduce cost the method was scaled down to use only 10μCi of [^{32}P]dCTP (NEG-013H from Dupont (UK) Ltd). The labelling mixture consisted of 25-125ng of heat denatured fragment DNA in 4μl of TE, 4.6μl of LS, O.4μl of 10mg/ml BSA, and 10 μCi of [^{32}P]dCTP. For whole plasmids this method worked best when plasmids had been cut into fragments using restriction endonucleases, extracted once with phenol/chloroform (1:1 v/v), precipitated with EtOH and resuspended to 100ng/μl in TE. For large plasmids in excess of 20 kb the labelling reaction was scaled up to 30 μCi [^{32}P]dCTP. The [^{32}P]-labelled DNA was separated from free [^{32}P]dCTP on a 1 ml G25-80 sephadex/TE column (Maniatis *et al.*, 1982) and denatured in boiling water for 5 minutes prior to addition to hybridization mixture.

2.4.11. Southern hybridizations. (Southern, 1975)

The transfer of DNA from agarose gels onto nitrocellulose was as described in Maniatis *et al.*, (1982). The nitrocellulose was purchased from Schleicher and Schuell and had a pore size of 0.45 μm. Prehybridisation and hybridisation of the filters was carried out according to Maniatis *et al.*, (1982). In homologous hybridization experiments filters were washed in several changes of 0.1xSSC/O.1%SDS at 65°C for 4 hours. In heterologous hybridization experiments washing conditions of increasing stringency were used, which usually involved gradual lowering of SSC concentration from 2xSSC at room temperature.

2.4.12. Colony hybridizations. (Grunstein and Hogness,1975)

Colonies were lifted from agar plates by placing a circle of sterile dry nitrocellulose onto the surface. The nitrocellulose was allowed to wet for 30-60 seconds. The position of each filter was marked using a series of pinpricks through the filter into the agar. The filter was then lifted off cleanly and treated as below. The agar plates were replaced in the 37°C incubator for 6-8 hours to regenerate the colonies. Lysis of the cells was effected by following procedure 1 in Maniatis *et al.*, (1982) in which the filters are firstly placed on Whatman 3MM paper soaked in denaturation solution (O.5M NaOH, 1.5M NaCl) for five minutes, then secondly on 3MM paper soaked in neutralising solution (1.5M NaCl, 0.5M Tris-HCl pH 8) for a further five minutes. After allowing to dry at room temperature, each filter was baked at 80°C for 2 hours. The filters were prewashed overnight in 3xSSC/O.1 SDS at 65°C to remove cellular debris. DNA hybridisation was carried out as for Southern hybridisation experiments.

2.4.13. Northern hybridisation.

Total RNA was run on 1.8% formaldehyde denaturing gels and transferred to nitrocellulose as described in Maniatis *et al.*, (1982). Prehybridisation, hybridisation and washing conditions were as described in Thomas (1983). When RNA 'dot blots' were performed the procedure outlined in Thomas (1983) was used.

2.5. Nucleotide sequencing.

2.5.1. Ml3 clone preparation.

The dideoxy chain termination method (Sanger *et al.*,1977; Messing *et al.*,1981) was used to determine the nucleotide sequences reported here. DNA fragments for sequencing were ligated into the appropriately cut replicative form of M13 mp18 or mp19 (Norrander *et al.*,1983), which were purchased from Pharmacia, and transformed into competent *E. coli* JMl05 or JM109 cells. 0.1 ml of competent cells were mixed with a solution of DNA (1-5μl) and left on ice for 40 minutes before being heat-shocked at 42°C for 2 minutes and returned to ice. 0.2 ml of exponentially growing JM109 or JM105 cells in 2xTY medium were added to provide a lawn. To 3 ml of H-Top agar (kept at 45°C) was added 25μl X-Gal(25mg/ml in dimethylformamide), 25μl IPTG (25mg/ml in water) and the cells. After a brief inversion, the cells were plated out on 2xTY plates (2xTY media containing 1.5% (w/v) bactoagar), and the top-agar allowed to set. The plates were inverted and incubated at 37°C overnight.

2.5.2. Preparation of template.

A template for sequencing was obtained by picking a white plaque with a toothpick and using it to innoculate 1.5 ml of 2xTY in a 20 ml test tube which contained a 1 in 100 dilution of an overnight culture of JM109 or JM105 . This culture was grown vigourously for 6 hours at 37°C. The 1.5 ml culture was transferred to an 1.5 ml Eppendorftube and the cells pelleted by spinning for 10 minutes in a microfuge (12,000xg). l ml of the supernatant was transferred to a fresh tube with care not to disturb the pellet. The supernatant was mixed with 250μl of 20% (w/v) polyethylene glycol 6000, 2.5 M NaCl and left to stand at room temperature for 15 minutes. The viral particles were pelleted by spinning for 10 minutes in a microfuge and the supernatant removed by aspiration. The tubes were respun to ensure complete removal of the polyethylene glycol and the pellet was resuspended in 400μl of TE buffer. The aqueous phase was extracted once with phenol/chloroform (l:l v/v) and once with chloroform/isoamyl alcohol (24:1 v/v). The DNA was not vortexed, but shaken by hand with care taken not transfer any of the organic phase though into the next extraction. The single-stranded DNA was ethanol precipitated, washed with 80% EtOH/H_2O (v/v) and dried *in vacuo*. The DNA was finally dissolved in 20μl of lxTE by gently tapping the side of the Eppendorf, and stored at -20°C.

2.5.3. Sequencing reactions.

Dideoxynucleotide sequencing reactions were carried out using two different enzyme systems, Klenow fragment and Sequenase.

2.5.3.1. Klenow dideoxynucleotide sequencing.

The protocol as outlined in the Amersham M13 cloning and sequencing manual was followed with the following modifications.

(a) the nucleotide analogue 7-deazo dGTP was substituted for dGTP to reduce secondary structure which can cause 'band compressions' on sequencing gels (Mizusawa *et al.*, 1986).

(b) the radiolabel was deoxyadenosine 5'-(alpha-[^{35}S])thio triphosphate, triethyl ammonium salt (650 Ci/mmole;8 mCi/ml) purchased from Amersham (Biggin *et al.*,1983).

(c) The elongation reactions were carried out at 50^{o}C. as suggested by Ornstein and Kashdan.

(d) The formamide dye mix was freshly made from BRL nucleic acid grade formamide stored at
-20^{o}C.

(e) 50 cm buffer gradient gels were used.

2.5.3.2. Sequenase dideoxynucleotide sequencing.

Sequenase, a modified T7 DNA polymerase (Tabor and Richardson, 1987) was purchased as part of a kit from Cambridge Bioscience. Sequencing reactions were carried out according to the 'Sequenase' guide (United States Biochemical Corporation, 3rd Edition). Buffer gradient gels were used.

2.5.4. Using M13 clones as a source of single stranded radiolabelled DNA.

ssDNA probes were made using the method outlined in detail in Aldea *et al.*, 1988, except that [^{32}P]dCTP (NEG-013H from DuPont (UK) Ltd) was used as the radiolabelling isotope.

2.6. Conjugating transfer of plasmids.

A variety of different procedures were used to transfer plasmids between different *E. coli* strains and from *E. coli* to *Rb. sphaeroides*. Though these methods have been described in Hunter (1988) and Hunter and Turner (1988) a selection of procedures used during the course of work for this thesis are presented.

2.6.1. Conjugating transfer of plasmids from *E. coli* to *E.coli.*

10 ml overnight *E. coli* cultures were grown under selective conditions. The cells were washed once with LB and resuspended to A680 = 10. The *E. coli* cultures were mixed in 1:1 ratio and 30μl placed on a well dried LB agar plate. After 6 hours at 37^{o}C, the mating mixture was harvested and spread out on suitable selective plates.

2.6.2. Triparental mobilisation of pSUP202 from *E. coli* strain C600::Tn5 to *E. coli* HB101 using helper plasmid pRK2073.

In outline the pSUP202 clone of choice is transformed into C600:: Tn5 cells. Then the helper plasmid pRK2073 was used to transfer pSUP202 clones (some now bearing Tn5) into HB101. Before the triparental mating was set up, 10 ml cultures of each of the three *E. coli* strains were grown overnight. The cells of each culture were washed with LB and resuspended in 100μl. Half of each of the three cultures was mixed in a 1.5 ml eppendorf tube and placed on a well dried LB agar plate. After 9 hours at 37^{o}C the mixture was harvested into 3 mls of LB, and 100μl aliquots spread on 30 LB agar plates using selection for a high level of streptomycin resistance (500μg/ml), kanamycin and chloramphenicol.

2.6.3. Conjugating transfer of plasmids from *E. coli* to *Rb. sphaeroides.*

When pSUP202 was used as the vector it could be transferred from *E. coli* 17-1 or SM10 to *Rb. sphaeroides* directly. Since each of these strains possesses the transfer functions of the conjugating plasmid RP4 which is immobilised in the chromosome. Usually a 1 cm long smear of donor *E. coli* from a freshly streaked selective plate was placed on the surface of a well dried LB agar plate. Then 25μl of a freshly grown *Rb. sphaeroides* culture resuspended to A680 = 10 was placed on top of the *E. coli* streak, and left for 6 hours at 32^{o}C. The mixture was then harvested in 100μl of M22^{+} and spread out on suitably selective M22^{+} plates. When bchl^{-} or other nonphotosynthetic mutants were used as recipients regained capacity for photosynthesis was selected for by placing the plates in anaerobic conditions in the light (see section 2.3.4.).

Photosynthetic transconjugants were produced with an efficiency of around $1x10^{-4}$ per recipient (Hunter and Turner, 1988). A similar system was used when transferring the broad host range vector pNH2 from *E. coli* SM10 to *Rb. sphaeroides* (Hunter and Turner, 1988).

2.6.4. Triparental conjugating transfer of plasmids from *E. coli* to *Rb. sphaeroides*.

When the *Rb. sphaeroides* gene library in *E. coli* DH5 was used it was necessary to utilize a triparental system to transfer the bank to *Rb. sphaeroides* mutants. pRK2073 was the preferred helper plasmid.
10 ml cultures of DH5[pRK2073] and *Rb. sphaeroides* were grown, washed in LB and resuspended to A680 = 10. A frozen 20µl aliquot of the *Rb. sphaeroides* gene bank in pSUP202 was thawed and washed once with LB to remove glycerol, and resuspended in 5µl of LB. The three were mixed in the proportion 1:1:50, *E. coli*: *E. coli*: *Rb. sphaeroides*, and 30µl placed on a well dried LB plate. After 9 hours at 32°C the mixture was harvested, spread on a $M22^+$ agar plate and photosynthetic selection applied using anaerobic jars as described in section 2.3.4. Photosynthetic transconjugants were produced with an efficiency of around $5x10^{-5}$ per recipient (Hunter and Turner, 1988).

2.6.5. Generation of random and directed *Rb. sphaeroides* transposon Tn5 mutants.

Two methods were used to generate Tn5 mutations in *Rb. sphaeroides*.

2.6.5.1. Random transposon Tn5 mutagenesis.

A culture of *E. coli* 17-1[pSUP202l] grown overnight in 10 mls of LB medium, was washed and resuspended to A680 = 10. A 10 ml culture of *Rb. sphaeroides* grown in $M22^+$ CAA was also washed in LB and resuspended to A680 = 10. The two were mixed in a 1:50, *E. coli*: *Rb. sphaeroides* ratio and a 100µl of this mixture placed on a well dried LB agar plate. After 6 hours at 32°C the mixture was harvested and plated out on 20 $M22^+$ agar plates containing neomycin. After 5 days growth at 32°C, the plates were usually placed in anaerobic jars (section 2.3.4.) for a further 4 days to help distinguish between wild-type and non-photosynthetic *Rb. sphaeroides* colonies. Colonies of putative mutants were restreaked and subjected to spectral analysis. Neomycin resistant transconjugants of *Rb. sphaeroides* were produced with an efficiency of around $2x10^{-6}$ per recipient (Hunter, 1988).

2.6.5.2. Directed transposon Tn5 mutagenesis of *Rb. sphaeroides.*

Plasmid 'miniprep' DNA was prepared from colonies of *E. coli* HB101[pSUP202::Tn5] (see section 2.6.2.). Each plasmid bears Tn5 inserted at a different position. Plasmids were transformed into *E. coli* 17-1. 10 mls of a 2 day old culture of *Rb. sphaeroides* wild-type was resuspended to A680 = 10. A 1 cm streak of each *E. coli* 17-1[pSUP202::Tn5] clone was placed on a well dried LB agar plate and a 30 µl aliquot of *Rb. sphaeroides* placed on top. After 6 hours at 32°C each mixture was placed in 100 µl of M22+ and spread on a M22+ Nm agar plate. After 5 days at 32°C the plates were placed in anaerobic jars in the light for a further 4 days. Non-photosynthetic mutant colonies were restreaked and subjected to spectral analysis. Neomycin resistant transconjugants of *Rb. sphaeroides* were produced with an efficiency of around $1x10^{-5}$ per recipient (Hunter, 1988).

2.7. Spectral methods of pigment characterisation.

2.7.1. Absorption spectra.

Absorption spectra were obtained on a Perkin-Elmer 554 spectrophotometer. Initially 80 ml *Rb. sphaeroides* cultures were grown as described in section 2.3.2. The cells were pelleted in a Beckman JA.14 rotor for 5 minutes, and the following procedures performed.

2.7.1.1. Absorption spectra of culture supernatants.

1 ml of the supernatant was placed in a 1 ml disposable plastic curvette. The absorption spectrum was recorded from 800 - 350nm.

2.7.1.2. Absorption spectra of antenna complexes in whole cells.

5% of a cell pellet was resuspended in a minimal amount of liquid (100 µl) then mixed with 10 mls of glycerol to obtain a translucent solution. An absorption spectrum was recorded in the range 900 - 750nm.

2.7.1.3. Absosrption spectra on acetone/methanol extracts of whole cells.

10% of a cell pellet in a minimal amount of liquid (100 µl total volume) was vortexed in the dark with 1 ml of acetone/methanol 7:2 (v/v) in a 1.5 ml Eppendorf tube.The cell debris was spun down at 12,000 rpm for 5 minutes in a bench Microfuge. The supernatant was removed and kept in the dark at 4°C. until needed. An absorption spectrum was recorded from 900 - 300 nm using glass curvettes.

2.7.1.4. Absorption spectrum of pigments in diethyl ether.

Acetone/methanol extractions of cell pigment were performed as in section 2.6.1.3. The supernatant was placed in a glass bijou bottle with 5 g of solid NaCl and the bottle shaken. Then 2 mls of diethyl ether was added, and the bottle extensively shaken and allowed to settle. The top diethyl ether layer was removed. The absorption spectrum was recorded in the range of 800 - 300nm. Glass curvettes were used.

2.7.2. Fluorescence emmission spectra.

Fluorescence emmission spectra were obtained on a Perkin-Elmer MPF-44A flourescence spectrophotometer. Guidance was taken from data kindly supplied by Dr. W.R. Richards (Simon Fraser University, Canada) to find the maximal excitation wavelength for each intermediate of bchl synthesis tested. Fluorescence emission spectra of acetone/methanol and diethyl ether extracted pigments (prepared as in section 2.6.1.3. and 2.6.1.4.) were recorded from 500 - 800nm.

2.8. High speed infrared photography to detect highly fluorescent mutant phenotypes.

Plates of putative mutants of *Rb. sphaeroides* were photographed using infrared photographic techniques to find highly fluorescent colonies. The phenomenon of high fluorescence is observed when mutants lack the ability to transfer energy through the light harvesting apoproteins to the reaction centre. The resulting energy is released as infrared light (Youvan *et al.*,1983). M22+ agar plates carrying well spaced 7 day old colonies were placed inverted on a large petridish containing copper sulphate solution. The petridishes were illuminated from below using a standard art work light box, shielded so that only blue light that passed through the copper sulphate filter was present. A Praktika TL 1000 camera was loaded in the dark with

Kodak High Speed Infrared Film (2481). After focusing on the *Rb. sphaeroides* colonies the camera lens was covered with a Schott RG780 infrared filter, so only infrared fluorescence was detected, and not the blue exciting light. The film was exposed for 20 seconds at f5.6. The film was processed according to manufacturers instructions. Colonies of high fluorescence phenotype appeared dark on the negative when compared to wild-type colonies.

2.9. S1 nuclease RNA transcript mapping.

A method of RNA transcript mapping which utilizes M13 probes, sodium trichloroacetate solvent, dideoxy sequencing ladders and S1 nuclease was used as described in Aldea *et al.*,(1988).

Table 2.1 ***E. coli*** **strains**

Strain	Description	Source
JM105	del(*lac-pro*AB), *thi*, *gyr*A96 *end*Al, *hsd*R17, *rel*Al, *sup*E44, (rk-, mk-), -[F', *tra*D36, *pro*AB, *lac*Iq Z del M15	Pharmacia Ltd.
JM109	*rec*A- (*rec*Al, *end*Al, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, lambda-, del(*lac-pro*AB), {F', *tra*D36, *pro*AB, *lac*Iq, *lac*Z del M15}	Stephen Mayes
DH5	a derivative of DH1, F-, *rec*Al, *end*Al, *gyr*A96, *thi*, *hsd*R17 (rk- mk+), *sup*E44, *rel*Al, lambda-	Neil Hunter
DH5 ∝	del*lac*Z delM15, *end*A1 *rec*A1 *hsd*R17 (rk- mk+) *sup*E44, *thi*-1, lambda-, *gyr*A96, *rel*A1, F-, del(*lac*ZYA-*arg*F) U169.	Stephen Mayes
17-1	*Pro*, *res*-, *mod*+, RP4 2-Tc::Mu-km:: Tn5 integrated into chromosome.	Simon *et al.*, (1983)
Sm10	a derivative of C600 : *rec*A, *thi*, *thr*, *leu*. RP4-2-Tc::Mu integrated into the chromosome; kan^R	Simon *et al.*, (1983)

HB101	F$^-$, *hsd*S20 (*r*B$^-$, *m*B$^-$), *sup*E44, *ara*14, lambda$^-$, *gal*K2, *lac*Y1, *pro*A2, *rps*L20, *xyl*-5, *mtl*.1, *rec*A13.... Restriction: (*r*B$^-$, *m*B$^-$), *mcr*A(+), *mcr*B(-).	Neil Hunter
C600::Tn5	*sup*E44 *thi*-1, *leu*B6, *lac*Y1, *ton*A21, -.Restriction:(*r*k+, *m*k+), *mcr*A(-) *mcr*B(+).	Neil Hunter

Table 2.2 **Plasmids**

Plasmid	Description	Source or reference
pNH2	Sm^R, Tc^R derivative of RSF1010; 8.05 kb *Eco* RI - *Ava* I fragment of RSF1010 ligated to 1.4 kb *Eco* RI - *Ava* I fragment of pAT153 bearing gene for Tc^R.	Hunter and Turner (1988)
pSUP202	Amp^R, Tc^R, Cm^R, mob+, tra-, col El replicon.	Simon *et al.*, (1983)
pJW1	Amp^R, 12.2 kb *Bam* HI fragment bearing *Rb. sphaeroides puf* operon cloned into pBR322.	J.C.Williams
pUC18/19	pBR322/mp19/18 derivative vector Amp^R, lacZ, lac:,	Yanisch-Perron *et al.*,(1985)
M13mp18/19	M13 derivative with 54 bp polylinker	Pharmacia Ltd. Yanisch-Perron *et al.*,(1985)

pRK2073	Sp^R; tra^+, mob^+, colM. El replicon.	Daniels/ Leong *et al.*, (1982)
pSUP2021	pSUP202::Tn5, Amp^R, Tc^R, Cm^R, Km^R.	Simon *et al.*, (1983)

Table 2.3 ***Rb sphaeroides*** **media**

1.	M22+	g/litre
	KH_2PO_4	3.6
	$K_2HPO_4.3H_2O$	3.0
	Sodium Lactate (70% w/v solution)	2.0
	$(NH_4)_2SO_40.5NaCl$	0.5
	SolutionC	20 mls
	Sodium succinate	4.6
	Sodium glutamate	0.27
	Aspartic acid	0.04

pH to 7.6. Autoclave

2.	Solution C	g/litre
	Nitrilotriacetic acid	10
	$MgCl_2.6H_2O$	24
	$CaCl_2.6H_2O$	3.34
	$EDTANa_2$	0.124
	$ZnCl_2$	0.261
	$FeCl_2.4H_2O$	0.251
	$MnCl_2.4H_2O$	0.09
	$(NH_4)MO_7O_{24}.4H_2O$	0.009
	$CuCl_2.2H_2O$	0.008
	$Co(NO_3)_2.6H_2O$	0.012
	H_3BO_3	0.006

Stored at -20°C.

3.	1OOOx Vitamins stock solution	mg/litre
	Nicotinic acid	1000
	Thiamine	500
	p-aminobenzoic acid	100
	Biotin	10

Filter sterilized, stored at 4°C.

CHAPTER 3

Construction of a physical map of the photosynthetic cluster of *Rb. sphaeroides*

3.1 Introduction

Rb. sphaeroides was the principle member of the *Rhodospirillaceae* family used in the biochemical determination of bacteriochlorophyll and haem biosynthesis pathway (Lascelles, 1978; Saunders, 1978; Jones, 1978; Castlefranco and Beale, 1981). In the early 1970's the discovery of the gene transfer agent by Marrs made *Rb. capsulatus* accessible to genetic mapping, which quickly lead to an increasing variety of genetic techniques (Scolnik and Marrs, 1987). In 1983 Taylor *et al.*, reported that the genes for carotenoid and bchl biosynthesis were linked in a 45 kb cluster whose extremities were marked by the *puh*A and *puf* operons encoding light harvesting (LHI) and reaction centre (RC) polypeptides. Although genes essential for photosynthesis were found to be clustered in *Rb. capsulatus* at the start of this work no similar photosynthesis gene clusters had been reported and it was not known if this was a common feature among photosynthetic bacteria. Here a cluster bearing genes for bchl biosynthesis and RC and LHI polypeptides has been isolated from *Rb. sphaeroides.*

3.2 Results

3.2.1. Isolation and characterisation of bacteriochlorophyll, carotenoid and reaction centre-less mutants of *Rb. sphaeroides*

A number of mutants of *Rb. sphaeroides* with lesions in bchl biosynthesis were available (C.N. Hunter, unpublished results). N5, N6, N1 and N22 were all generated using N-Methyl-N'-nitro-N-nitro-guanidine (NTG) mutagenesis (Hunter and Turner, 1988). Nevertheless, a number of bchl$^-$, crt$^-$ and RC$^-$ mutants which had previously been described in *Rb. capsulatus* and *Rb. sphaeroides* were not available (Saunders, 1978; Taylor *et al.*, 1983), so in order to increase the range of *Rb. sphaeroides* mutants available both NTG and random Tn5 mutagenesis techniques were employed (Hunter and Turner, 1988; section 2.6.5.1.). Over 100 NTG and Tn5 generated mutants were isolated which could not photosynthesise. These were characterized using the spectroscopic techniques described in section 2.7. Many of the bchl$^-$ mutants had similar phenotypes to those already isolated. However, one Tn5 generated mutant, T127, exhibited a new bchl$^-$ phenotype. Several NTG and Tn5 generated crt$^-$ mutants displaying blue green and green phenotypes were isolated.

One NTG generated mutant, NF57, lacking RC and LHI absorption characteristics and possessing a high fluorescence phenotype was also isolated.

3.2.2. Characterisation of bchl⁻ mutants

Mutants defective in bchl biosynthesis accumulate bchl derivatives, and in many cases excrete these derivatives into the growth medium. Lascelles and Richards (1969) have identified the intermediates accumulated by a number of bchl⁻ mutants of *Rb. sphaeroides*. In *Rb. capsulatus*, Marrs and co-workers assigned each different bchl⁻ mutant to a lesion in a specific *bch* gene (Taylor *et al.*, 1983; Biel and Marrs, 1983). In this work absorption spectra of cell-free culture supernatant and from cells extracted with acetone: methanol were recorded for each bchl⁻ mutant (see section 2.7.), and the spectra compared to those obtained in earlier studies (Richards and Lascelles, 1969; Pudek and Richards, 1975). Fluorescence emission spectra (see section 2.7.) were also obtained for each bchl⁻ mutant and compared to those obtained from bchl⁻ mutants of *Rb. capsulatus* (Biel and Marrs, 1983, and unpublished data kindly supplied by Dr. W.R. Richards, Simon Fraser University, Canada). Absorption spectra for the bchl⁻ mutants N6, N5, N22 and T127 are presented in figure 3.1. Fluorescence emission data for bchl⁻ mutants N5, N6, N22 and T127 is presented in Table 3.1. The bchl intermediate identified for each bchl⁻ mutant is also listed in Table 3.1. It should be noted that although the identity of each excreted intermediate is probably characteristic of a specific lesion in bchl biosynthesis, many bchl⁻ mutants also contain smaller amounts of intermediates characteristic of preceding biosynthetic steps to the one blocked. Also this method of identification does not distinguish between intermediates which have similar fluorescence and absorption properties but are chemically different. For example the use of HPLC techniques has shown that both monovinyl and divinyl derivatives of Mg phaeporphyrin a_5 monomethyl ester are present in the bchl⁻ mutant N5 (B. White and Dr W.T. Griffiths, personal communication). It is clear from this result and the alternative pathways described by Pudek and Richards (1975)(shown in figure 1.2), that bacteriochlorophyll biosynthesis may not be a directly linear pathway. Each bchl biosynthesis enzyme or enzyme complex may be able to utilize a number of tetrapyrrole intermediates with varying specificity. Further work needs to be done to characterize fully the bchl intermediates excreted by bchl⁻ mutants in order to identify tetrapyrroles present in low concentration and those hidden by similar absorption spectrum profiles.

3.2.3. Identification of NF57

The NTG mutant NF57 only shows absorption peaks at 800 and 850 nm in spectra of whole cell suspensions in glycerol (section 2.7.) (see figure 3.1). Combined with the observation that NF57 had a highly fluorescent phenotype it was concluded that this mutant lacked LHI and RC complexes. This was confirmed using SDS-PAGE when only bands corresponding to LHII α and β polypeptides were observed (Hunter and Grondelle, 1988). It should be noted that mutants with lesions in either *puh*A or *puf* operons in *Rb. capsulatus* display the same phenotype as NF57 (Youvan *et al.*,1982, 1983). Since its isolation NF57 has been used in a number of studies on biophysical and structural properties of LHII complexes within the membrane environment (van Dorssen *et al.*, 1988; Vos *et al.*, 1988; Hunter *et al.*, 1988b).

3.2.4. Isolation of clones carrying bchl biosynthesis genes from a gene library in pNH2

The preparation of a gene bank of *Rb. sphaeroides* DNA in *E. coli* DH5 has been described in Hunter and Turner (1988). This work employed the RSF1010 derivative pNH2 as a cloning vector. The pNH2 bank consists of 8000 colonies of average insert size 5.5 kb, f=0.999 (Maniatis *et al.*, 1982). It was found that although pNH2 could be stably maintained in *Rb. sphaeroides*, the presence of an insert of cloned *Rb. sphaeroides* DNA lead to the rapid integration of the recombinant into the chromosome (results not shown). To overcome this a three stage procedure was adopted to identify clones carrying insert DNA which could restore *Rb. sphaeroides* bchl$^-$ mutants to wild type phenotype.

1. The gene library was divided into 10 sublibraries of around 500 colonies. Each was transferred from *E. coli* to a given *Rb. sphaeroides* bchl$^-$ mutant.

2. Any sublibrary which produced a significant number of photosynthetic Sm^R transconjugants was divided into 10 further sublibraries of 100 colonies. Each of these sublibraries was transferred to the appropriate *Rb. sphaeroides* bchl$^-$ mutant.

3. In the final round each one of the one hundred colonies from the second stage sublibrary which produced photosynthetic Sm^R transconjugants was transferred to the appropriate *Rb. sphaeroides* bchl$^-$ mutant. All transfers were performed as described in section 2.6.

Only two clones which restored bchl- mutants to wildtype phenotype were successfully isolated from the pNH2 gene bank despite several attempts with all the bchl- mutants listed in Table 3.1. The clone pSC16 contained an insert of 4.0 kb (figure 3.2H) and restored mutant N5 to wild type phenotype. The second clone, pSC124, contained an insert of 4.8 kb and restored mutant N1 to wild type phenotype. The N1 mutant and pSC124 form the basis of Chapter 5.

3.2.5. Construction of a *Rb. sphaeroides* gene library in vector pSUP202

A further library was constructed in the mobilizable, pBR325 derivatives pSUP202 (Simon *et al.*, 1983). Fragments of DNA produced by partial digestion with the restriction endonuclease *Taq* I were size fractionated on a 1.5-5M NaCl salt gradient as described in Hunter and Turner, (1988). Fragments in the range 10-14 kb were ligated into the *Cla* I site of pSUP202 which had been treated with calf intestinal alkaline phosphatase, and the ligation mix transformed into *E. coli* DH5. As over 40,000 colonies were obtained a number of libraries each containing 2500 clones of average insert size 11 kb were produced. Only 800 colonies are needed to give f=0.999 (Maniatis *et al.*, 1982).

3.2.6. Isolation of clones carrying bchl biosynthesis genes from the library in pSUP202

pSUP202 cannot stably maintain itself in *Rb. sphaeroides* (Scolnik and Marrs, 1987; Hunter and Turner, 1988). So in order to isolate clones carrying bchl biosynthesis genes it was necessary to adapt a 2-stage isolation procedure, similar to that employed for the pNH2 genebank.

Three clones were isolated; pSCN5-1, pSCN6-1 and pSCN22-1 which restored bchl- mutants N5, N6 and N22 respectively to wild type phenotype. Restriction maps of pSCN5-1, pSCN6-1 and pSCN22-1 showing the position of *Eco* RI, *Hind* III, *Pst* I and *Bam* HI sites are presented in figure 3.2BCF. pSCN6-1 was characterized further using restriction endonucleases *Nru* I and *Sma* I to check the order of *Pst* I and *Eco* RI fragments that were uncut by the other three restriction endonucleases initially used (results not shown). It was noted that pSCN5-1 and pSCN6-1 overlapped and shared 6 kb in common. This was confirmed using Southern hybridization techniques (results not shown).

These clones were used as probes to find overlapping clones in the gene library. A number of fragments were therefore prepared from pSCN5-1, pSCN22-1 and at a later stage pSCN22-11. The fragments that were used are indicated by shading on figure 3.2; they are all internal fragments and bear no pSUP202 DNA. Each fragment was

labelled with [^{32}P]dCTP (section 2.4.10.) and used to probe four 15cm diameter nitrocellulose filters lifted from agar plates bearing around 1000 colonies. Colonies which hybridized to each probe were toothpicked and screened again for positive hybridisation. On average 12 positive colonies were isolated with each probe. Plasmid DNA was prepared from each colony, digested with a number of restriction endonucleases and subjected to agarose gel electrophoresis. Each probe yielded 3-4 digest patterns which differed from the parent clone. In each case the clone with the largest insert which showed least restriction endonuclease digest similarity with the parent clone was chosen for detailed restriction endonuclease mapping. Four clones pSCN5H-1, pSCN6-20, pSCN22-11 and pSCN22-15 were isolated by this method, and mapped using *Eco* RI, *Hind* III, *Pst* I and *Bam* HI restriction endonucleases (figure 3.2ADEG). Overlaps indicated by restriction map similarity were checked using Southern hybridisation techniques. pSCN22-15 was found to possess the same 0.6 kb *Eco* RI - *Pst* I fragment found in plasmid pJW1. pJW1 (isolated and characterized by Dr Joanne Williams) contains a 12.2 kb *Bam* HI fragment of *Rb. sphaeroides* DNA which contains the *puf* operon. pSCN5H-1, pSCN5-1, pSC16, pSCN6-1, pSCN6-20, pSCN22-11, pSCN22-1 and pSCN22-15 form the basis of the restriction endonuclease map of the *Rb. sphaeroides* photosynthetic gene cluster which is 46 kb in length (figure 3.3).

3.2.7. Mapping the position of bchl$^-$ and RC$^-$ lesions on the photosynthetic gene cluster of *Rb. sphaeroides*

All the clones indicated in figure 3.3 were transformed into *E. coli* SM10 and then transferred to a number of mutants unable to synthesize either bacteriochlorophyll, or reaction centre complexes (listed in Table 3.1). The ability of each clone to restore wild type phenotype to each mutant is recorded in Table 3.2. In figure 3.3 *bch*E, *bch*B, *bch*A and *bch*C and *puh*A are positioned according to data from Table 2.4. Two subclones of pSCN5H-1 bearing the 1.68 kb *Bam* HI and 2.7 kb *Pst* I restriction fragments (shaded on figure 3.2A) called pSCB6 and pSCP4 respectively were constructed in the vector pSUP202. On transfer both pSCP4 and pSCB6 restored *Rb. sphaeroides* mutant NF57 to wild type phenotype.

3.2.8. Hybridization of cloned *Rb. sphaeroides* photosynthesis genes with DNA from other bacteria.

Genomic DNA was prepared from the following organisms using the method described in section 2.4.2.; *Rb. sphaeroides* 8253, *Rb. capsulatus* Z1, *Rhodospirillum rubrum* S1, *E. coli* DH5. Southern blots were prepared and heterologous

hybridization was performed in 6xSSC, 5xDenhardts, 0.5% SDS, 100µg/ml denatured calf thymus DNA at 65°C, overnight. The filters were washed in 2xSSC/0.1% SDS at room temperature for 1 hour, followed by 1xSSC/0.1% SDS at room temperature for 1 hour and exposed overnight. The results of probing genomic Southern blots with DNA fragments marked 1-6 on figure 3.3 are presented in figure 3.4.

Beatty and Cohen, (1983) used DNA restriction fragments from the photosynthesis gene cluster of *Rb. capsulatus* to probe a number of photosynthetic organisms. Their heterologous hybridization experiments led them to conclude that there was little homology even between different *Rhodobacter* species. However, in this work it is clear, that for the hybridization conditions used, *Rb. sphaeroides* and *Rb. capsulatus* show distinct homology. Significant, though in some cases fainter bands are also seen in *Rhodospirillum rubrum* tracks.

3.3. Discussion

The positions of the previously characterized *puh*A and *puf* operons (section 1.6), including direction of transcription are marked on the 46 kb photosynthetic gene cluster (figure 3.3). The *Rb. sphaeroides* mutant NF57 was restored to wild type phenotype by pSCN5H-1, pSCN5-1 and subclones pSCB6 and pSCP4. The two fragments carried on clones pSCB6 and pSCP4 include the upstream region of the *puh*A gene, which suggests that the lesion in NF57 is in the promoter region of this gene. Lesions in the *puh*A gene of *Rb.capsulatus* have been shown to produce RC/LHI-less mutants (Youvan *et al.*,1982, 1983). This observation indicates that the H subunit plays an essential part in stabilising LHI and RC associations. Study of the RC-H polypeptide using site directed mutagenesis techniques is needed in order to understand fully its role in the photosynthetic membrane.

Both *puh*A and *puf* operons are found in identical positions at either end of the photosynthetic cluster of *Rb. capsulatus* (Taylor *et al.*, 1983). The locations of the *bch*B, *bch*E, *bch*C and *bch*A genes are indicated only approximately on the *Rb. sphaeroides* cluster, but nevertheless are found in corresponding positions on the *Rb. capsulatus* map. The six carotenoid biosynthesis genes isolated by Pemberton and Harding (1986) from *Rb. sphaeroides* occupy a region of similar size to that in *Rb. capsulatus*. The restriction map presented in figure 3.3 show little similarity with the *crt* cluster previously published (Pemberton and Harding, 1986), though the different strains used (NCIB 8253 and RS6258) could account for this. The *Rb. sphaeroides* *puc*AB operon encoding LHII α and ß polypeptides (Kiley and Kaplan, 1987; Ashby *et al.*, 1987) was not found in the 46 kb photosynthetic cluster of *Rb. sphaeroides*. The *Rb. capsulatus* *puc*AB operon is also not found in the main 45 kb photosynthetic

cluster (Youvan and Ismail, 1985) and has recently been found to lie over 1000 kb away on the chromosome map, assembled from a series of sequential cosmid clones (Dr Joanne Williams, personal communication).

The similarity in size and gene order, together with the homology shown in heterologous hybridization experiments, suggest that the photosynthesis gene clusters of *Rb. sphaeroides* and *Rb. capsulatus* share a common evolutionary origin. Indeed, comparisons of 16S rRNA and cytochrome c sequences have grouped these bacteria closely together (Ambler, 1985).

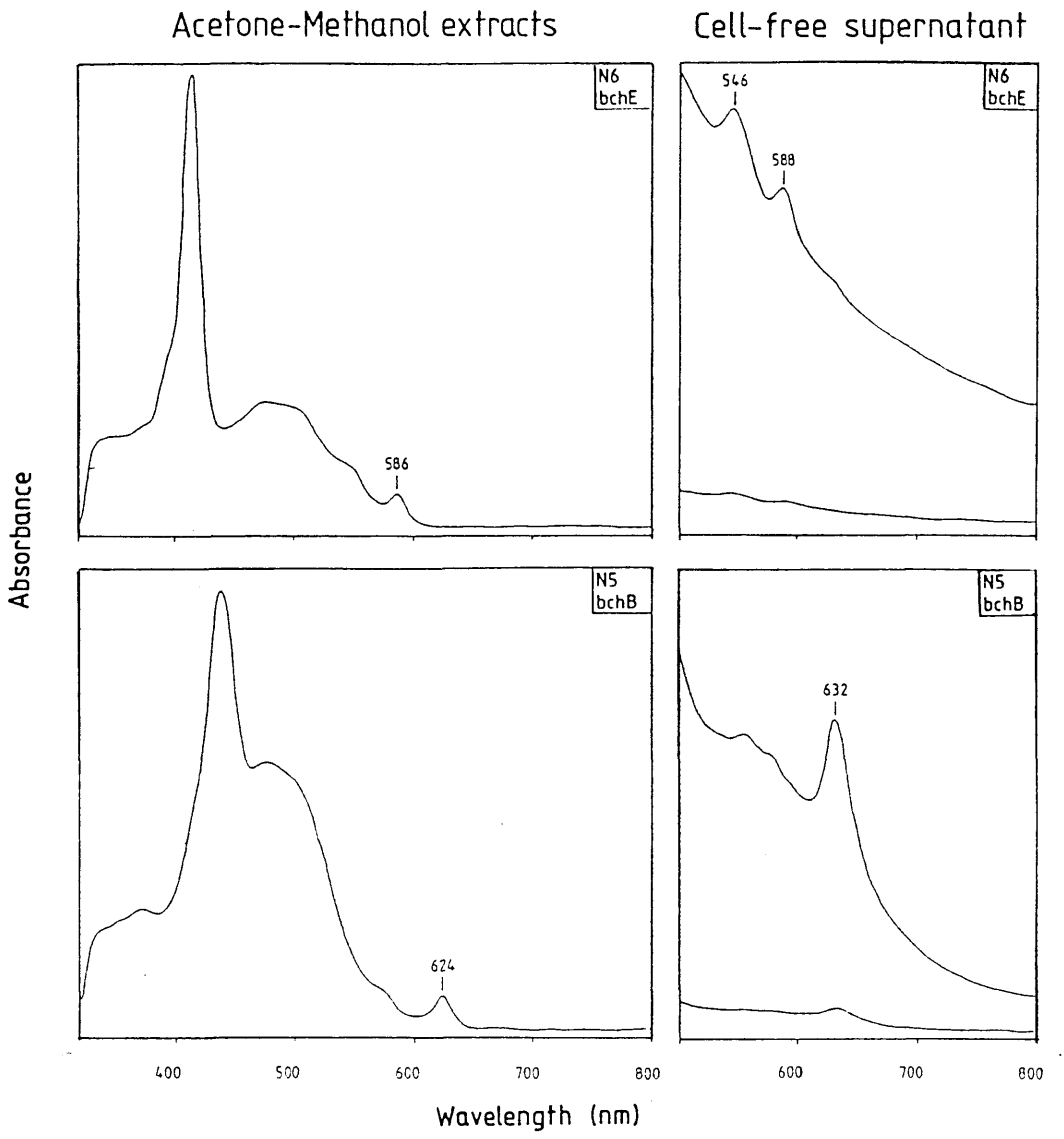


Figure 3.1

Absorption spectra of acetone-methanol cell extracts and cell free supernatant for *Rb. sphaeroides* bchl mutants N6 and N5. The vertical axis is equivalent to 1 absorbance unit full scale, except in the second scan of the cell free supernatant spectra were the vertical scale is 0.1 full scale. The far red absorption maxima of each bacteriochlorophyll intermediate are labelled. In both cases the absorption spectra between 900-700 nm for whole cells in glycerol was featureless due to lack of reaction centre and light harvesting complexes.

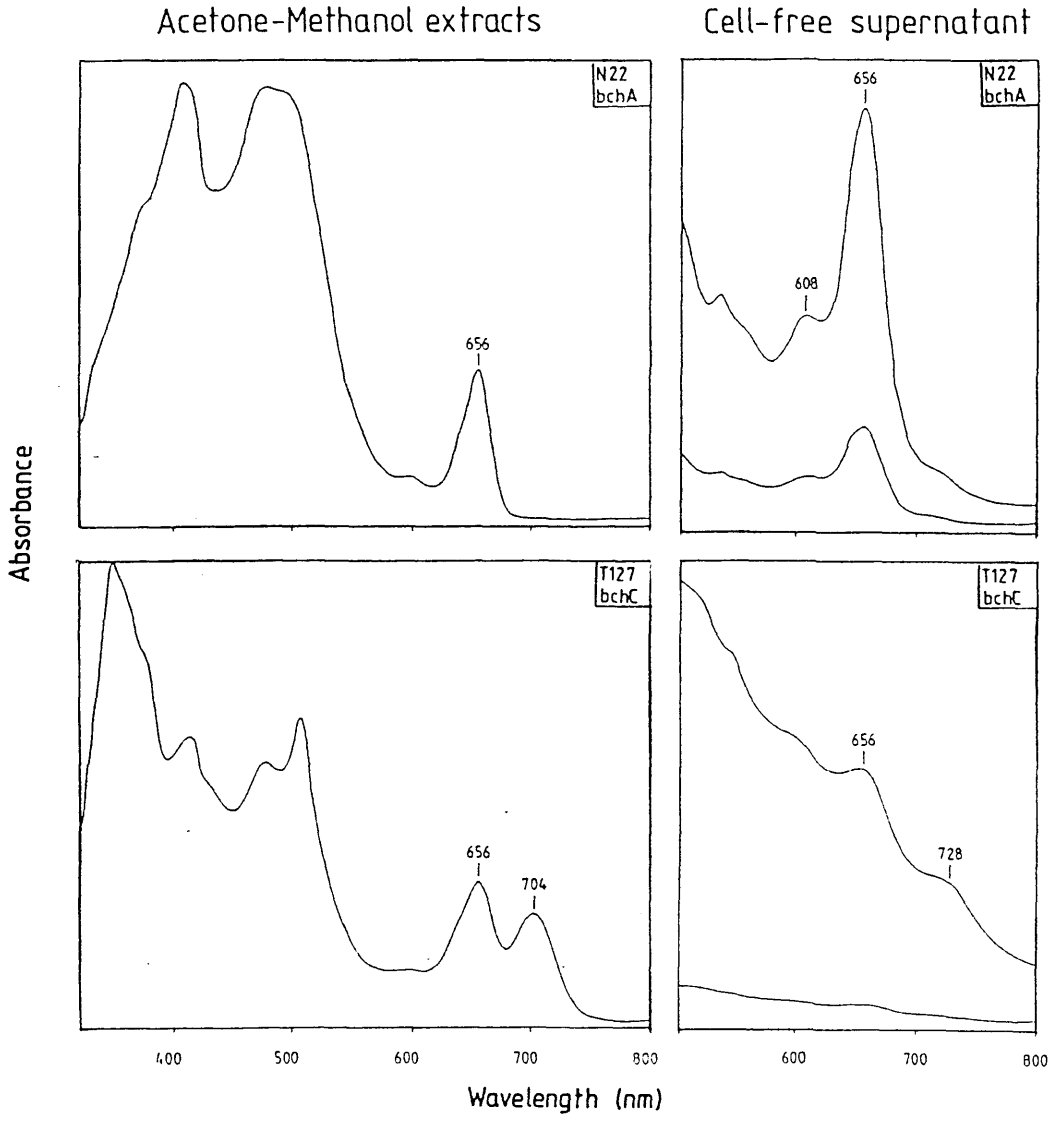


Figure 3.1 (continued)

Absorption spectra of acetone-methanol cell extracts and cell free supernatant for *Rb. sphaeroides* bchl mutants N22 and T127. The vertical axis is equivalent to 1 absorbance unit full scale, except in the second scan of the cell free supernatant spectra were the vertical scale is 0.1 full scale for T127 and 0.2 full scale for N22. The far red absorption maxima of each bacteriochlorophyll intermediate are labelled. In both cases the absorption spectra between 900-700 nm for whole cells in glycerol was featureless due to lack of reaction centre and light harvesting complexes.

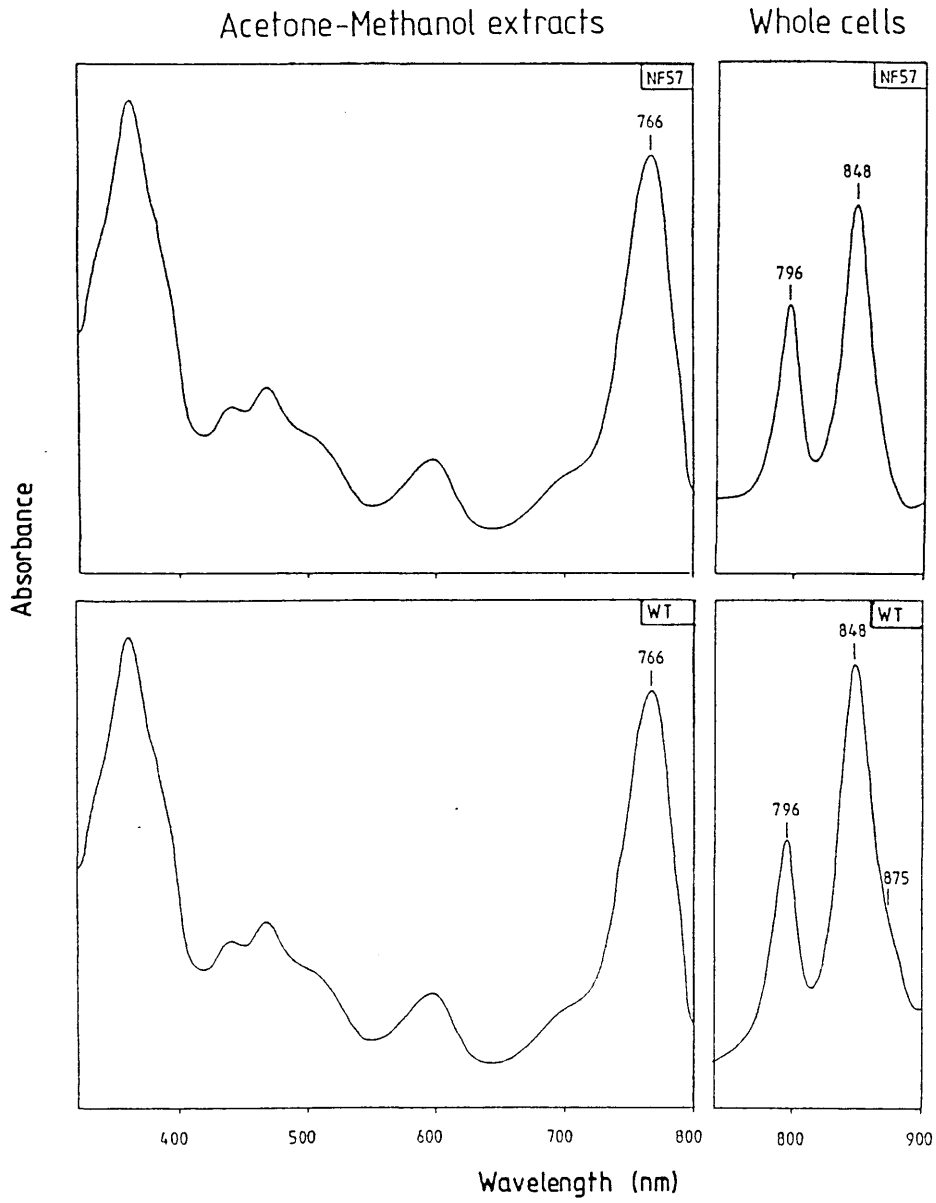


Figure 3.1 (continued)

Absorption spectra of acetone-methanol cell extracts and whole cells in glycerol for wild type *Rb. sphaeroides* and the mutant NF57. The vertical axis is equivalent to 1 absorbance unit full scale. The far red absorption maxima are labelled. In both cases the absorption spectra of the cell free supernatant between 800-500 nm was featureless.

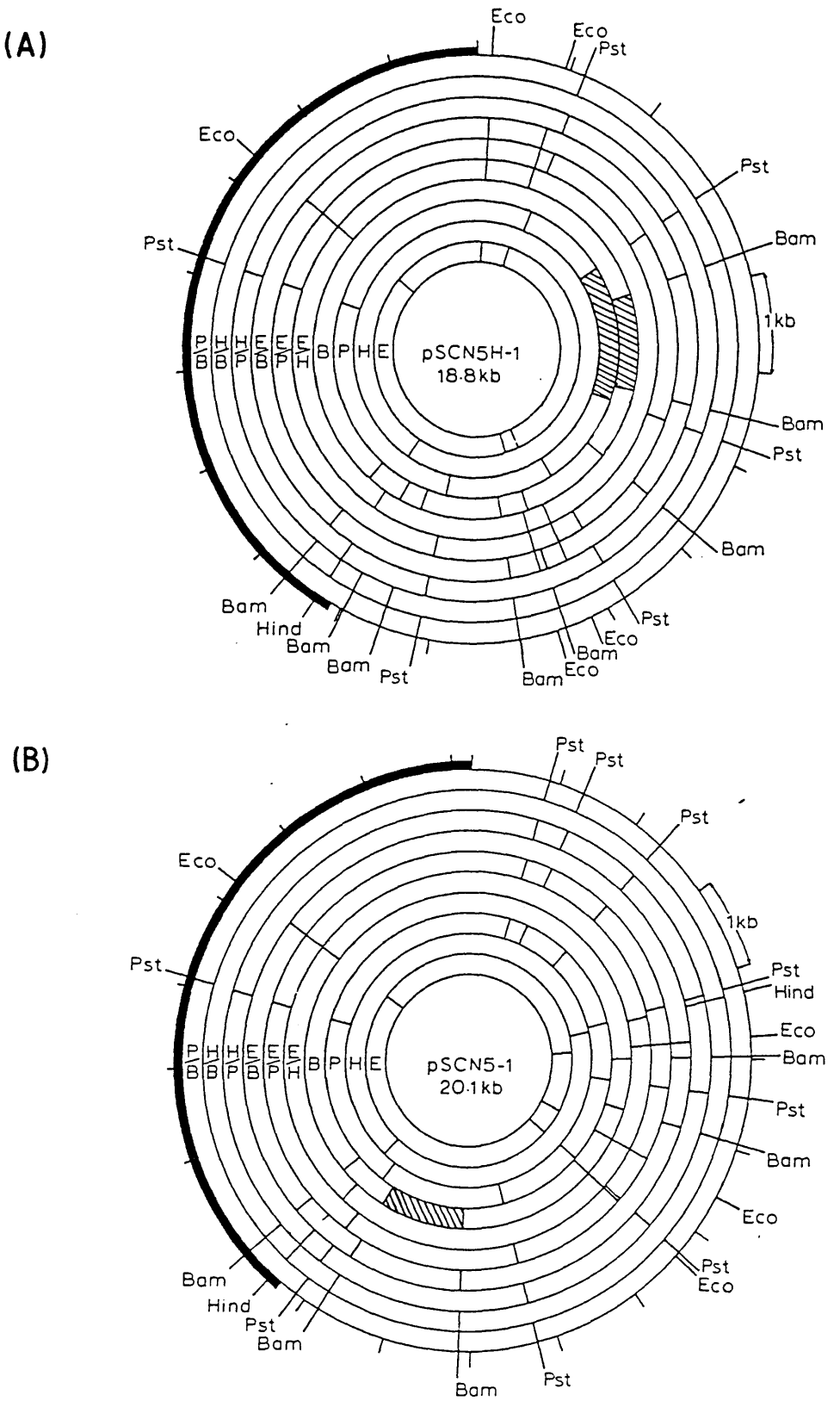
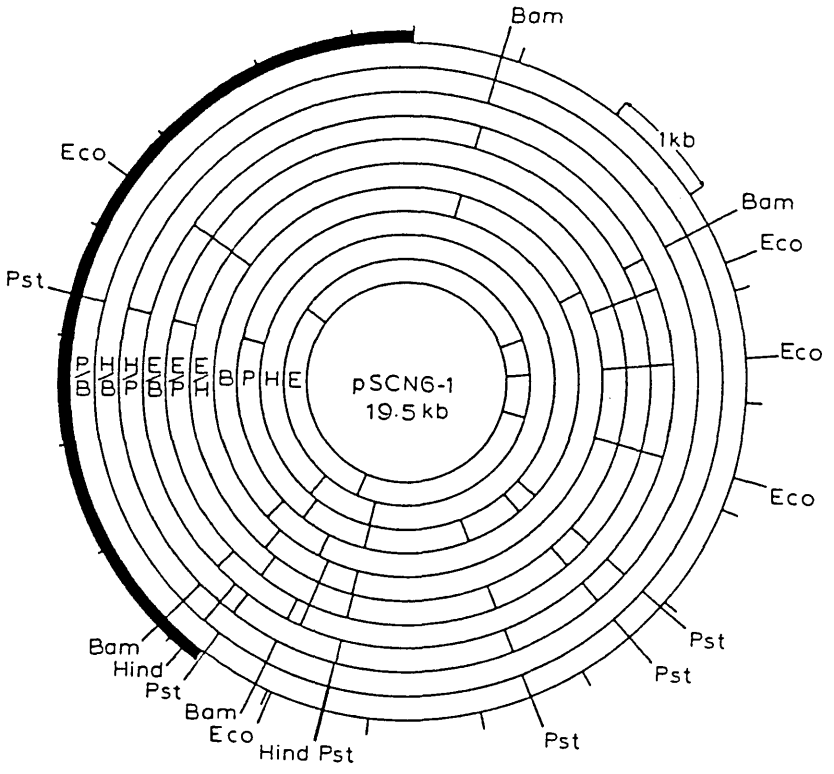


Figure 3.2AB

Restriction maps of plasmids pSCN5H-1 and pSCN5-1 showing *Eco* RI, *Hind* III, *Pst* I and *Bam* HI sites. The fragment of pSCN5-1 used to isolate pSCN5H-1 from the pSUP202 library is indicated by shading (lines). The shaded (lines) fragments on pSCN5H-1 indicated the subcloned fragments described in section 3.2.7. The vector, pSUP202, is shaded (block).

(C)



(D)

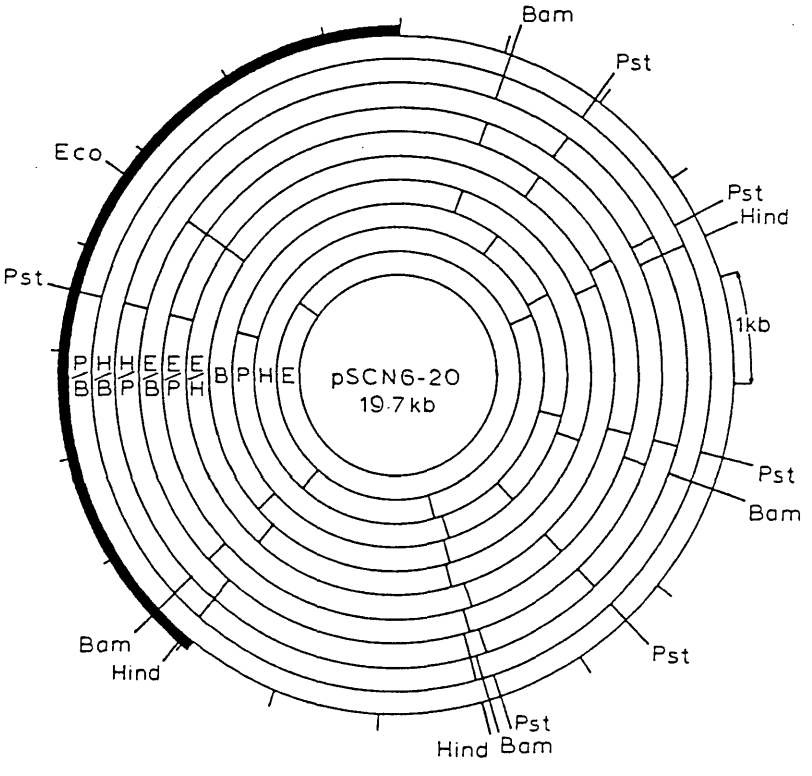
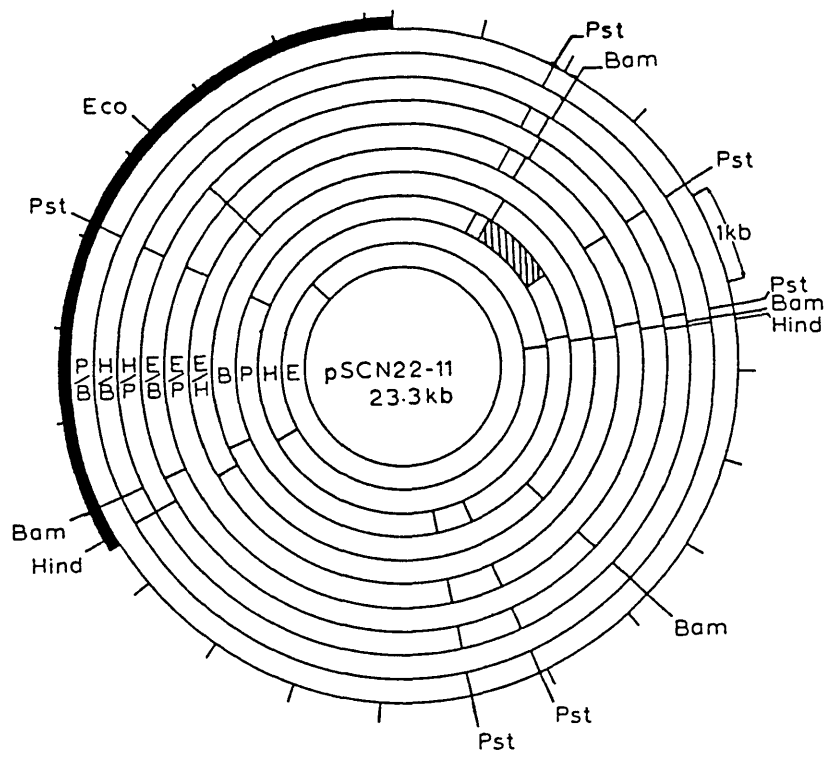


Figure 3.2CD

Restriction maps of plasmids pSCN6-1 and pSCN6-20 showing *Eco* RI, *Hind* III, *Pst* I and *Bam* HI sites. The vector, pSUP202, is shaded (block).

(E)



(F)

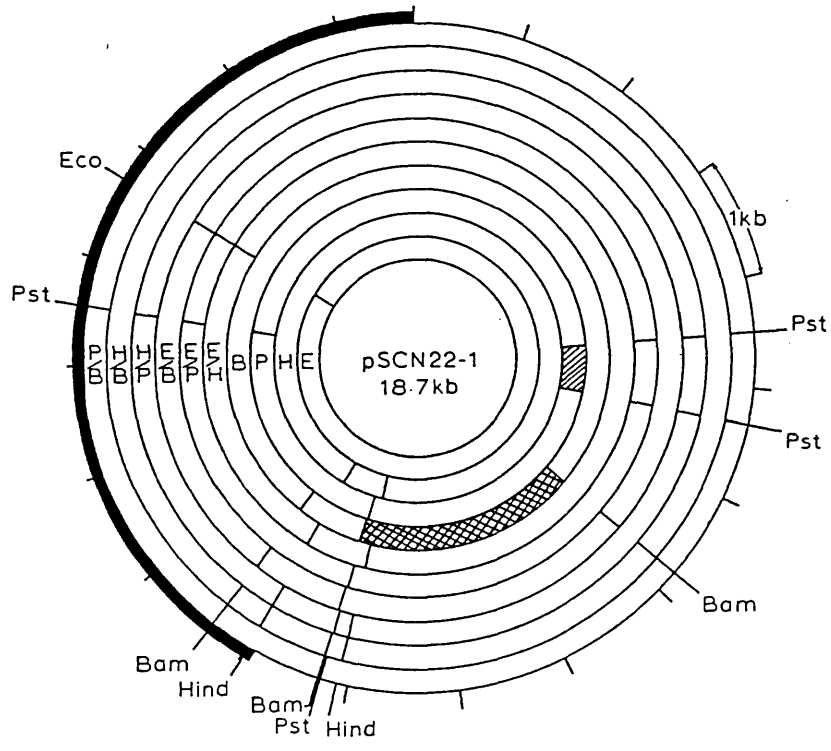
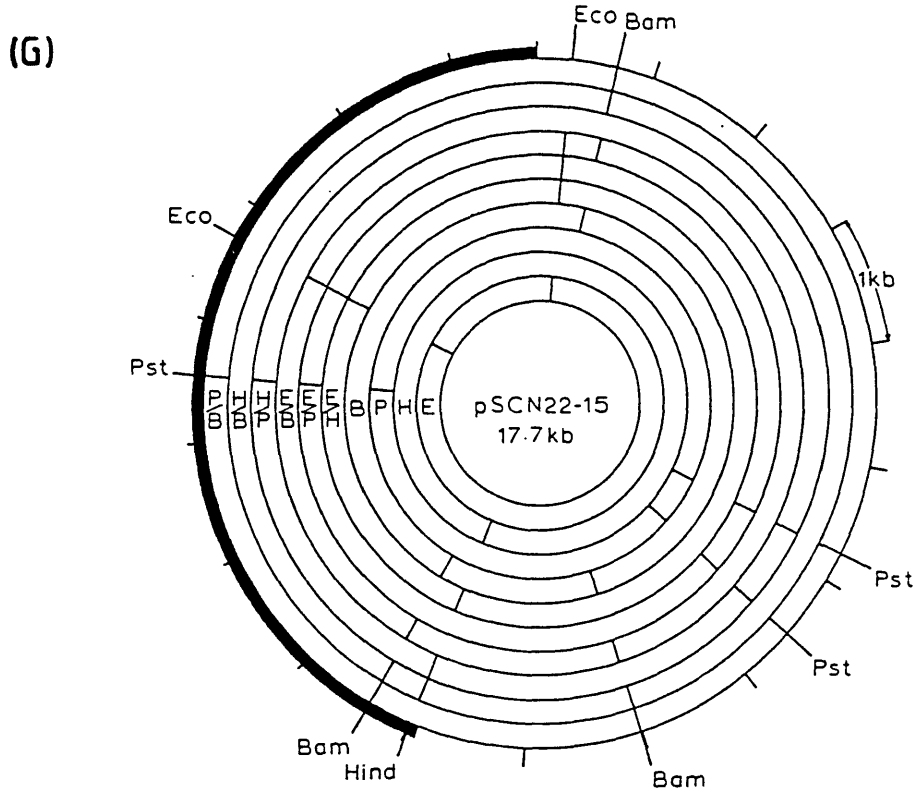


Figure 3.2EF

Restriction maps of plasmids pSCN22-11 and pSCN22-1 showing *Eco* RI, *Hind* III, *Pst* I and *Bam* HI sites. The fragment of pSCN22-11 used to isolate pSCN6-20 is indicated by shading (lines). The fragment of pSCN22-1 used to isolate pSCN22-11 is indicated by shading (cross-hatch). The fragment of pSCN22-1 used to isolate pSCN22-15 is indicated by shading (lines). The vector, pSUP202, is shaded (block).



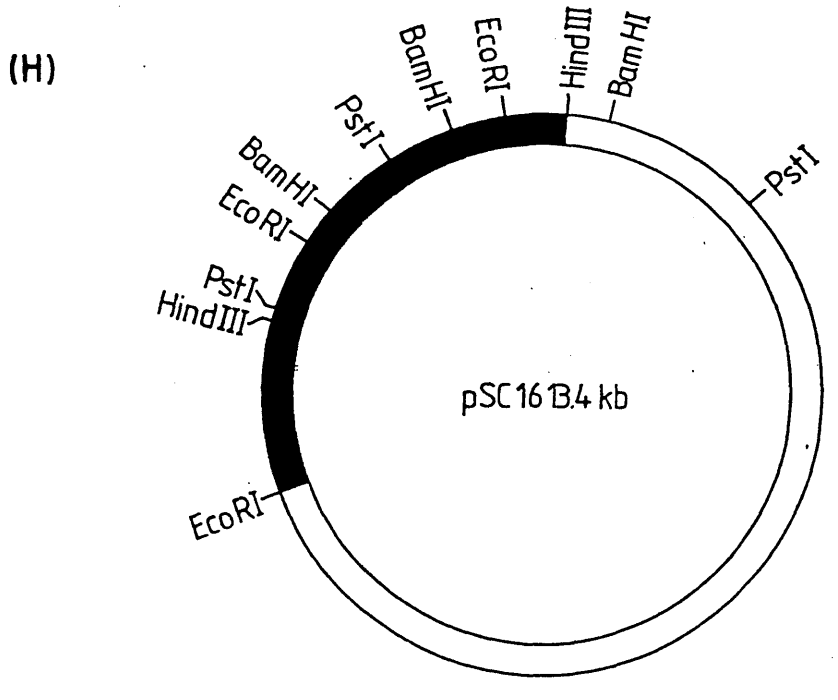


Figure 3.2GH

Restriction maps of plasmids pSCN22-15 and pSC16 showing *Eco* RI, *Hind* III, *Pst* I and *Bam* HI sites. The vector, pSUP202, is shaded (block) for pSCN22-15. The vector, pNH2, is unshaded in pSC16..

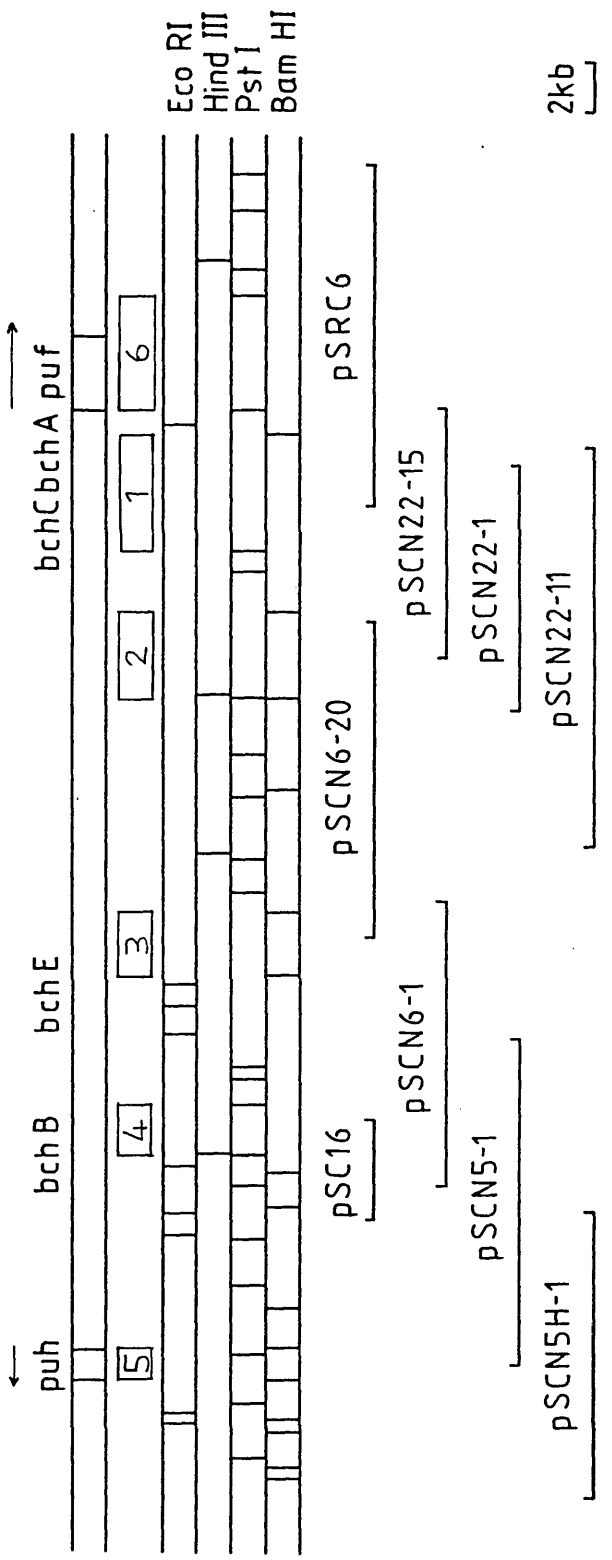


Figure 3.3

A restriction and complementation map of 45 kb of the *Rb. sphaeroides* chromosome bearing the photosynthesis cluster. Positions of the previously characterized *puh* and *puf* genes are marked along with the approximate positions of *bch*B, *bch*E, *bch*A and *bch*C derived from data in Table 3.2. The positions of clones used in this work are indicated. The restriction fragments used for the heterologous hybridization experiments in figure 3.4 are marked 1-6.

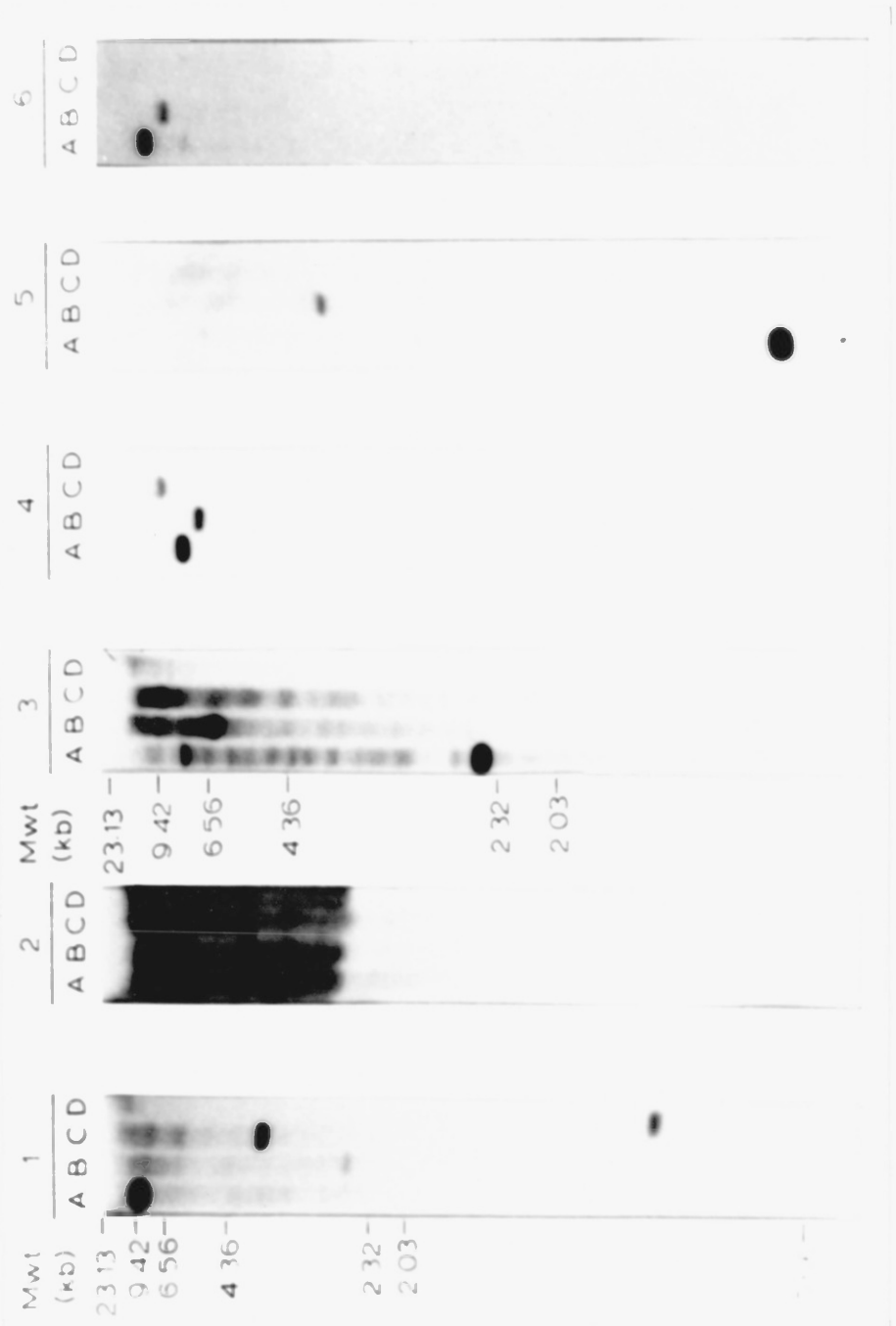


Figure 3.4

Southern blot analysis of the genomic DNA from other bacteria. 10μg of DNA from (A) *Rb. sphaeroides*, (B) *Rb. capsulatus*, (C) *Rs. rubrum*, (D) *E. coli* were digested with *Bam* HI. The blots were probed with fragments 1-6 indicated in figure 3.3.

Mutant	Absorption (nm) Solvent: Acetone/ methanol	Absorption (nm) Solvent: Diethyl ether	Fluorescence emission (nm) Excitation wavelength (nm)	Fluorescence emission (nm) Solvent: Acetone/ methanol	Fluorescence emission (nm) Solvent: Diethyl ether	Possible bacteriochlorophyll intermediate
N6	586	588	423	590	592	Mg protoporphyrin mono-methyl ester
N5	624	622	450	629	625	Mg divinyl phaeoporphyrin a_5 monomethyl ester
N22	656	656	427	668	660	2-desvinyl-2-hydroxyethyl-chorophyllide *a*
T127	704	708	370	718	718	2-desacetyl-2-hydroxyethyl-bacteriochlorophyllide *a*

Table 3.1

Major absorption and fluorescence emission peaks of acetone/methanol and diethyl ether extracts of whole cells of bchl mutants.

Strain	pSCN5H-1	pSCN5-1	pSCN6-1	pSCN6-20	pSCN22-11	pSCN22-1	pSCN22-15	pSRC6	pSC16
N6	-	-	+	-	-	-	-	-	-
N5	-	+	+	-	-	-	-	-	+
N22	-	-	-	-	+	+	+	+	-
T127	-	-	-	-	+	+	+	-	-
NF57	+	+	-	-	-	-	-	-	-

+ denotes wild type phenotype restored; - denotes mutant phenotype retained

Table 3.2

The restoration of bacteriochlorophyll and reaction centre-less *Rb. sphaeroides* mutants to wild type phenotype by clones presented in figure 3.3.

CHAPTER 4

Site directed transposon Tn5 mutagenesis of the *Rb. sphaeroides* photosynthetic gene cluster

4.1. Introduction.

Transposons have been widely used in the genetic analysis of a number of organisms because they possess the property of inserting into DNA and thus, disrupting genes. Two basic groups of techniques have been used to produce transposon (Tn) insertions into bacterial genomic DNA (reviewed by de Bruijn, 1987; Noti *et al.*, 1987).

1. Random transposon mutagenesis: the transposon is introduced into the bacterial species of interest via transformation, transduction or conjugation, followed by a transposition event and selection for a transposon carried antibiotic resistance marker.
2. Site directed transposon mutagenesis: transposon insertion into DNA segments cloned in plasmids in *E. coli* is followed by re-introduction of the specific transposon mutated segments into their original bacterial background. The wild type gene or region is then replaced by its transposon mutated homologue via double recombination.

The transposon Tn5 has been widely used in both these processes. Tn5 is 5818 bp in length and consists of two inverted repeats of 1535 bp, flanking a unique region which contains three genes organised in one operon that confers upon certain hosts resistance to the antibiotics kanamycin, neomycin, bleomycin and/or streptomycin. The entire nucleotide sequence of Tn5 has been determined and the locations of the coding regions of antibiotic resistance genes, transposition functions and sites involved in transposition have been mapped (reviewed in Noti *et al.*, 1987).

4.1.1. Random Tn5 mutagenesis

Random Tn5 mutagenesis of *Rb. capsulatus* and more recently *Rb. sphaeroides* has employed the vector pSUP2021. pSUP2021 is a pBR325 derivative, a Col E1 replicon which is unstable in a number of Gram negative organisms (Simon *et al.*, 1983; de Bruijn, 1987) and also carries Tn5. Random Tn5 mutagenesis has been used to produce mutations in a number of *Rb. capsulatus* genes including *crt*, *puf*, and *puc* genes (Kaufman *et al.*, 1984), a fructose utilizing gene *fru*A (Daniels *et al.*,1988), aminolevulinate synthase gene *hem*A (Wright *et al.*,1987) and several *nif* genes (Klipp *et al.*, 1988). pSUP2021 has been used to generate random Tn5 mutants in the *puf* operon (Hunter, 1988) and in *bch* and *crt* genes (Chapter 3).

4.1.2. Site directed transposon mutagenesis

Site directed transposon mutagenesis has been widely used on a number of occasions in *Rb. capsulatus*. Youvan *et al.*, (1982) introduced a number of different transposon Tn7 insertions into the R prime plasmid pRPS404 which has a 50 kb insert containing the photosynthetic gene cluster of *Rb. capsulatus*. He assigned 24 Tn7 insertions to *Bam* HI and *Eco* RI fragments and described their phenotype, but produced no overall map. Zsebo and Hearst (1984) used transposon Tn5.7 and the same R prime plasmid pRPS404 and mapped 45 insertions into the *Rb. capsulatus* photosynthesis cluster.

pSUP202 has become widely used in Gram negative organisms as a 'suicide vector' for site directed Tn5 mutagenesis (reviewed by Noti *et al.*, 1987). In *Rb. sphaeroides* Falcon *et al.*, (1988) subjected two pSUP202 clones carrying Rubisco bisphosphate carboxylase/ oxygenase genes to Tn5 mutagenesis and mapped nine Tn5 insertions that produced carboxylase mutant phenotypes. Hunter (1988) used the previously characterised *puf* operon to show that pSUP202 could be used as a vector for site directed Tn5 mutagenesis in *Rb. sphaeroides*.

Here, five clones in pSUP202 were subjected to Tn5 mutagenesis and transferred to *Rb. sphaeroides*. These clones have already been shown to overlap and carry *bch*, *puh*A and *puf* genes (Chapter 3). The positions of each Tn5 insertion has been mapped and the mutant phenotype identified in each case.

4.2. Results

4.2.1. Tn5 insertion into pSUP202 clones

Five pSUP202 clones, pSCN5H-1, pSCN5-1, pSCN6-20, pSCN22-1 and pSCN22-15 were subjected to Tn5 mutagenesis as described in section 2.6.2. Ninety six *E. coli* colonies which showed resistance to neomycin (Tn5), and chloramphenicol and ampicillin (pSUP202) were chosen for each clone. Although Hunter (1988) used a triparental system to transfer pSUP202 from HB101 into *Rb. sphaeroides* this was found to be unreliable. Instead, 'miniprep' plasmid DNA was made from each of the 96 colonies and transformed into *E. coli* strain 17-1 which contains the transfer functions of RP1 immobilized in the bacterial genome. Transfer of the various Tn5 bearing clones from *E. coli* 17-1 to *Rb. sphaeroides* was performed as described in section 2.6.3. Each mating mixture was then spread on a M22+ Nm agar plate and incubated at 32°C in the dark for 5 days. The plates, each carrying an average of 200 Nm^R colonies, were then placed in anaerobic jars (section 2.3.4.) and illuminated for a further 3-4 days in order to see if any colonies had lost the ability to photosynthesis.

4.2.2. Characterisation of Tn5 mutant phenotypes

Following transfer from *E. coli* a number of Tn5 bearing plasmids produced a variety of bchl$^-$, crt$^-$ and PS$^-$ phenotypes in *Rb. sphaeroides*. Around 70% of the Tn5 bearing plasmids from each clone produced colonies of wild type phenotype only. These clones were assumed to have Tn5 inserted in the vector itself or in a non-essential region of the *Rb. sphaeroides* insert DNA or in a region close to the edge of the insert. Indeed one of the 'hot spots' for Tn5 insertion is the promoter region of the tetracycline resistance gene found on pSUP202 (Berg *et al.*, 1983). It was noted that for any given transfer, the number of colonies displaying a single mutant phenotype varied between 5% and 40%. Hunter (1988) found that 1 in 10 Nm^R colonies produced by site directed Tn5 mutagenesis of the *puf* operon of *Rb. sphaeroides* showed a mutant phenotype and also lacked pSUP202 consistent with double reciprocal crossover events. It should be noted that the proportion of kanamycin resistant transconjugants produced by double reciprocal crossover events in site directed mutagenesis has been found to vary according to the length of flanking DNA in *Rhizobium*. For example, when Tn5 was placed in the middle of a 4 kb DNA insert and transferred to *Rhizobium*, 1 out of 250 km^R transconjugants were the result of a double reciprocal crossover. In contrast when Tn5 was inserted into the middle of a 13 kb DNA insert 1 out of 12 of the km^R transconjugants were the result of a double reciprocal crossover event (Noti *et al.*, 1987).

One typical mutant *Rb. sphaeroides* colony was chosen from each plate and restreaked. Each *Rb. sphaeroides* Tn5 mutant was grown in semiaerobic culture (section 2.3.2.) and subjected to spectral analysis (section 2.7.). In general bchl-less mutants were identified solely according to data obtained from absorption spectra (see chapter 3 for details and Figure 4.1B). *Rb. sphaeroides* Tn5 mutants with lesions in carotenoid biosynthesis were identified partially from absorption spectra (see Figure 4.1B). To identify the mixtures of carotenoids present in each crt mutant a number were analysed further using TLC and HPLC techniques. The TLC work was performed by Professor Richard Cogdell (University of Glasgow, U.K.) and the HPLC work was done by Ann Connor and Dr. George Britton (University of Liverpool, U.K.). Using these results each carotenoid mutant phenotype was assigned to a carotenoid gene using nomenclature already established for *Rb. capsulatus* (Scolnik et al., 1980; Guiliano et al., 1988).

4.2.3. Mapping of transposon Tn5 insertions

Each *E. coli* donor clone which produced a Nm^R mutant phenotype in *Rb. sphaeroides* was used as a source of plasmid DNA which was mapped using restriction endonucleases. Initially, plasmid DNA was prepared from each clone by the miniprep method described in section 2.4. Each plasmid was digested with two restriction endonucleases, usually *Pst* I and *Bam* HI, as these enzymes cut *Rb. sphaeroides* DNA relatively frequently. Although bands with elevated molecular weights were fairly easy to spot on agarose gels, DNA was often transferred to nitrocellulose and probed with the 3.5kb *Hind* III fragment of Tn5. Figure 4.2A shows a typical mapping gel. To check that the positions mapped for each Tn5 insertion were correct and to ensure that no large rearrangement of flanking DNA had occurred genomic DNA was prepared from a number of *Rb. sphaeroides* Tn5 mutants. The position of the Tn5 insertion in each case was mapped using *Pst* I and *Bam* HI restriction endonucleases, in conjunction with Southern hybridization in which the blots were probed with fragments from the *Rb. sphaeroides* photosynthetic cluster. Figure 4.2B shows a typical autoradiogram. Figure 4.3 shows the position of each Tn5 insertion which produced a mutant phenotype on the clone of origin. Table 4.1 lists the mutant phenotype produced by each Tn5 insertion. Figure 4.4. shows all the transposon Tn5 insertions mapped onto the photosynthetic cluster of *Rb. sphaeroides*. Fourteen of the Tn5 insertions in the region covered by pSCN5-1 and pSCN6-1 were mapped and the mutant phenotypes produced characterized by Maliha Chaudri, these are clearly distinguished in Table 4.1..

4.3. Discussion

A large number of transposon Tn5 insertions which produce mutant *Rb. sphaeroides* phenotypes have been mapped within the photosynthetic gene cluster. Each bchl$^-$, crt$^-$ or PS$^-$ phenotype has been marked by more than one Tn5 insertion except in the case of *bch*G. Although the error in mapping Tn5 insertions varies from 100 - 300 bp depending on fragment size, the number and extent of Tn5 insertions which produce each phenotype help overcome the inaccuracies of mapping. In figure 4.5. a general map of *Rb. capsulatus* has been produced from the combined data from several papers (Taylor *et al.*, 1983; Biel and Marrs, 1983; Zsebo and Hearst, 1984; Guiliano *et al.*, 1988). The crt cluster of *Rb. capsulatus* has recently been characterized further using interposon mutagenesis (Guiliano *et al.*, 1988) and this data is used in preference to data previously obtained by mapping with the gene transfer agent (Yen and Marrs, 1976; Scolnik *et al.*, 1980).

4.3.1. The *crt* genes

In this work a *crt* cluster of similar size and composition to that of *Rb. capsulatus* has been shown in *Rb. sphaeroides*. Although Tn5 insertions which show a *crt*F mutant phenotype have not been isolated, an appropriate gap exists in the overall *Rb. sphaeroides* map which could accommodate this gene. Interposon mutagenesis techniques will be used to identify the *crt*F mutant phenotype.

The carotenoidless or blue-green mutant phenotypes of *crt*B and *crt*E can not be distinguished apart and occupy separate positions in the *crt* gene cluster of *Rb. sphaeroides*, similar to that found in *Rb. capsulatus*. Two Tn5 insertions were found to accumulate the carotenoid phytoene attributed by Zsebo and Hearst (1984) and Guiliano *et al.*, (1986) to lesions in the *crt*I gene. Recently Guiliano *et al.*, (1988) have shown that *crt*I and *crt*B form an operon transcribed in the order *crt*I-*crt*B, although they found no interposon insertion which produced *crt*I mutant phenotype. This work again shows that *crt*I is closely linked to *crt*B (called *crt*I/*crt*B on figure 4.4) in *Rb. sphaeroides*, although the order of *crt*I and *crt*B is different from that found in *Rb. capsulatus*..

The *crt*D and *crt*C genes occupy a position in *Rb. sphaeroides* similar to that found in *Rb. capsulatus*. Two transposon Tn5 insertions which produce a *crt*A mutant phenotype were positioned at the end of the *crt* cluster next to the *crt*I/B in *Rb. sphaeroides*, in a position similar to that found in *Rb. capsulatus*. Overall, this work is in good agreement with that on *Rb. capsulatus* showing that the *crt* genes of *Rb. sphaeroides* are clustered and show a similar order. In both cases the basis for identification of lesions in *crt* genes was precise measurement of the physical - chemical properties of the carotenoids produced by mutant strains. A cluster of six *crt* genes including *crt*A, *crt*B, *crt*C, *crt*D, *crt*E and *crt*F has already been described in *Rb. sphaeroides* (Pemberton and Harding, 1986), although in this case no crt characterization and identification was presented. Moreover, there is little agreement in restriction endonuclease sites, although this could be accounted for by strain differences. Nevertheless, the size and gene order is similar to that reported here.

4.3.2. The *bch* genes

In *Rb. capsulatus* Zsebo and Hearst (1984) identified three new *bch* genes, *bch*I, *bch*J and *bch*K within the photosynthetic cluster. None of the Tn5 mutants studied here showed a *bch*J or *bch*I phenotype. Moreover Guiliano *et al.*, (1988) showed that an interposon positioned 0.9 kb away from the *crt*A gene produced a mutant phenotype consistent with a lesion in *bch*D, not *bch*I. It should be noted that the discrimination between *bch*I and *bch*D phenotypes depends on the presence of a trace of pigment

absorbing at 590nm (Zsebo and Hearst, 1984) so confusion is likely to occur. Nevertheless, the Tn5 insertions reported here are ascribed to *bch*D which is shown in Figure 4.4. as a region 3 kb in length, following the nomenclature of Taylor *et al.*, (1983).

In *Rb. capsulatus* mutant phenotypes similar to that found for *bch*D also result from lesions in two further genes, *bch*H (Taylor *et al.*, 1983; Zsebo and Hearst, 1984) and *bch*K (Zsebo and Hearst, 1984). In *Rb. sphaeroides* two clusters of Tn5 insertions both showing *bch*D-like mutant phenotypes appear in positions similar to those in *Rb. capsulatus* and so they have been named *bch*H and *bch*K. The *bch*D and *bch*H gene products are thought to be involved in the magnesium chelation and methylation steps preceding Mg protoporphyrin monomethyl ester formation. Since it is not currently possible to distinguish between *bch*D, *bch*H and *bch*K on the basis of absorption spectra, and unlike later stages in bchl synthesis, it is possible that *bch*K gene product is also involved at this stage of the bacteriochlorophyll biosynthesis pathway.

Four Tn5 insertions producing a phenotype attributed in *Rb. capsulatus* to lesions in the *bch*E gene (Taylor *et al.*, 1983, Biel and Marrs, 1983), were mapped in a similar position in the *Rb. sphaeroides* gene cluster (M. Chaudri, unpublished). A single Tn5 insertion which produced a phenotype attributed to *bch*G in *Rb. capsulatus* (Taylor *et al.*, 1983; Biel and Marrs, 1983) mapped in a comparable position in the *Rb. sphaeroides* photosynthetic cluster (M. Chaudri unpublished).

A 1 kb cluster of Tn5 insertions showed a phenotype attributed in earlier work to mutant N5 (Coomber *et al.*,1987, Hunter and Coomber, 1988) and *bch*B mutants in *Rb. capsulatus* (Biel and Marrs, 1983). Zsebo and Hearst (1983) reported Tn5.7 insertion which they attributed to a lesion in *bch*F which accumulated a pigment absorbing at 630nm previously used by Biel and Marrs (1983) as a indication of a lesion in *bch*B. The work reported here has identified a similar pigment and we therefore revert to the earlier nomenclature of Biel and Marrs, (1983) and designate this *bch*B. The *bch*F mutant phenotype accumulates bchl intermediates absorbing at 730nm and 665nm (Taylor *et al.*, 1983), no similar mutant phenotype was found in this work.

In *Rb. capsulatus* lesions in the gene designated *bch*L are phenotypically similar to those in *bch*B although these genes are over 7 kb apart (Zsebo and Hearst, 1984). In *Rb. sphaeroides* two clusters of Tn5 insertions which produce similar phenotypes are found in the same positions, and so the second gene has also been called *bch*L. Although Zsebo and Hearst (1983) did not assign a function for the *bch*L gene product(s) it could well be involved along with the *bch*B gene product(s) in the conversion of Mg- 2,4- divinyl pheoporphyrin a5 monomethyl ester to chlorophyllide, which involves the addition of 4 hydrogen atoms. The protochlorophyllide

intermediate of this reaction has not been isolated perhaps indicating that the *bch*B and *bch*L gene products act together in a complex.

Two further *bch* genes, *bch*A and *bch*C have been located between the *crt* cluster and *puf* operon in *Rb. capsulatus* (Taylor *et al.*, 1983). Tn5 insertions producing phenotypes typical of lesions in *bch*C and *bch*A map in a similar position in *Rb. sphaeroides*. This agrees with the position of these genes obtained from complementation data (Chapter 3). Bowen and Pemberton (1985) positioned two *bch* genes which they called *bch*A and *bch*B on the same side of the *crt* cluster in *Rb. sphaeroides*. However as they did not describe the bchl intermediates accumulated by these lesions it is not clear if their data is comparable to that found in this work.

A cluster of Tn5 insertions which produce a high fluorescence phenotype mapped within the *puh*A gene which has been fully characterised in *Rb. sphaeriodes* (Williams *et al.*, 1986; Donohue *et al.*, 1986a). In *Rb. capsulatus*, mutants of similar phenotype have been attributed to lesions in the *puh*A gene (Youvan *et al.*, 1982; Youvan *et al.*, 1984).

It is clear that the two photosynthetic clusters of *Rb. sphaeroides* and *Rb. capsulatus* are remarkably similar in size and order of genes (figure 4.4 and figure 4.5).

Table 4.1

Plasmid	Insertion number	Genotype	Phenotype
pSCN5H-1	TH15 (G)	*puh*A, Nm^R	PS-, HFr
	TH18 (G)	*puh*A, Nm^R	PS-, HFr
	TB109	*bch*L, Nm^R	PS-, P622
	TB112	*bch*L, Nm^R	PS-, P622
	TB114	*bch*L, Nm^R	PS-, P622
	TB111	*bch*L, Nm^R	PS-, P622
pSCN5-1	TB359	*bch*H, Nm^R	PS-, bchl- *
	TB241	*bch*H, Nm^R	PS-, bchl-
	TB340	*bch*H, Nm^R	PS-, bchl- *
	TB239	*bch*H, Nm^R	PS-, bchl-
	TB223	*bch*H, Nm^R	PS-, bchl-
	TB355	*bch*H, Nm^R	PS-, bchl- *
	TB352	*bch*H, Nm^R	PS-, bchl- *
	TB217	*bch*H, Nm^R	PS-, bchl-
	TB343	*bch*H, Nm^R	PS-, bchl- *
	TB219 (G)	*bch*K, Nm^R	PS-, bchl-
	TB207	*bch*K, Nm^R	PS-, bchl-
	TB208	*bch*K, Nm^R	PS-, bchl-
	TB210	*bch*K, Nm^R	PS-, bchl-
	TB215 (G)	*bch*K, Nm^R	PS-, bchl-
	TB345	*bch*K, Nm^R	PS-, bchl- *
	TB203	*bch*B, Nm^R	PS-, P624
	TB329	*bch*B, Nm^R	PS-, P624 *
pSCN6-1	TB306	*bch*E, Nm^R	PS-, P586 *
	TB314	*bch*E, Nm^R	PS-, P586 *
	TB366	*bch*E, Nm^R	PS-, P586 *
	TB337	*bch*E, Nm^R	PS-, P586 *
	TB303	*bch*G, Nm^R	PS-, P760 *

pSCN6-20	TB61 (G)	*bch*D, Nm^R	PS-, bchl-
	TB66	*bch*D, Nm^R	PS-, bchl-
	TB58	*bch*D, Nm^R	PS-, bchl-
	TB59	*bch*D, Nm^R	PS-, bchl-
	TB65	*bch*D, Nm^R	PS-, bchl-
	TB64	*bch*D, Nm^R	PS-, bchl-
	TB56	*bch*D, Nm^R	PS-, bchl-
	TB57	*bch*D, Nm^R	PS-, bchl-
	TB55	*bch*D, Nm^R	PS-, bchl-
	TC51 (G)	*crt*A, Nm^R	PS+, brown
	TC52 (G)	*crt*A, Nm^R	PS+, brown
	TC72	?*crt*B, Nm^R	PS+, blue-green
	TC70 (G)	*crt*B, Nm^R	PS+, blue-green
	TC69	?*crt*B, Nm^R	PS+, blue-green
	TC67 (G)	*crt*I, Nm^R	PS+, blue-green
	TC68	?*crt*I, Nm^R	PS+, blue-green
	TC71 (G)	*crt*I, Nm^R	PS+, blue-green
pSCN22-1	TC44 (G)	?*crt*B, Nm^R	PS+, blue-green
	TC47	?*crt*B, Nm^R	PS+, blue-green
	TC40 (G)	*crt*C, Nm^R	PS+, green
	TC41 (G)	?*crt*D, Nm^R	PS+, green
	TC45	*crt*E, Nm^R	PS+, blue-green
	TC48	?*crt*E, Nm^R	PS+, blue-green
	TC46	?*crt*E, Nm^R	PS+, blue-green
	TC43	?*crt*E, Nm^R	PS+, blue-green
	TB36 (G)	*bch*A, Nm^R	PS-, P656
	TB39 (G)	*bch*A, Nm^R	PS-, P656
	TB37	*bch*A, Nm^R	PS-, P656

pSCN22-15	TC15 (G)	*crt*C, Nm^R	PS+, green
	TC20	*crt*D, Nm^R	PS+, green
	TC16	*crt*D, Nm^R	PS+, green
	TC18	*crt*D, Nm^R	PS+, green
	TC19	*?crt*D, Nm^R	PS+, green
	TC27 (G)	*crt*E, Nm^R	PS+, blue-green
	TC23 (G)	*?crt*E, Nm^R	PS+, blue-green
	TC29	*crt*E, Nm^R	PS+, blue-green
	TC24	*crt*E, Nm^R	PS+, blue-green
	TC30	*?crt*E, Nm^R	PS+, blue-green
	TC31 (G)	*crt*E, Nm^R	PS+, blue-green
	TC32	*crt*E, Nm^R	PS+, blue-green
	TB34	*bch*C, Nm^R	PS-, P704
	TB09	*bch*A, Nm^R	PS-, P656
	TB01	*bch*A, Nm^R	PS-, P656
	TB07	*bch*A, Nm^R	PS-, P656
	TB06	*bch*A, Nm^R	PS-, P656
	TB05	*bch*A, Nm^R	PS-, P656
	TB02	*bch*A, Nm^R	PS-, P656
	TB10	*bh*A, Nm^R	PS-, P656
	TB04 (G)	*bch*A, Nm^R	PS-, P656
	TB08 (G)	*bch*A, Nm^R	PS-, P656

Each Tn5 insertion is listed under plasmid of origin. The genotypes follow the nomenclature used for *Rb. capsulatus* (summerized in Scolnik and Marrs, 1987). *?crt* is used for carotenoid mutants whose intermediates have yet to be characterized. HFr indicates a high fluorescent phenotype (section 2.8). PS+/- indicates ability to grow photosynthetically in anaerobic conditions. bchl- means no bacteriochlorophyll intermediate could be detected. The number after P indicates the absorption maximum in acetone-methanol cell extracts for bchl mutants. (G) indicates those mapped using genomic DNA. * denotes those insertions mapped and characterized by M. Chaudri.

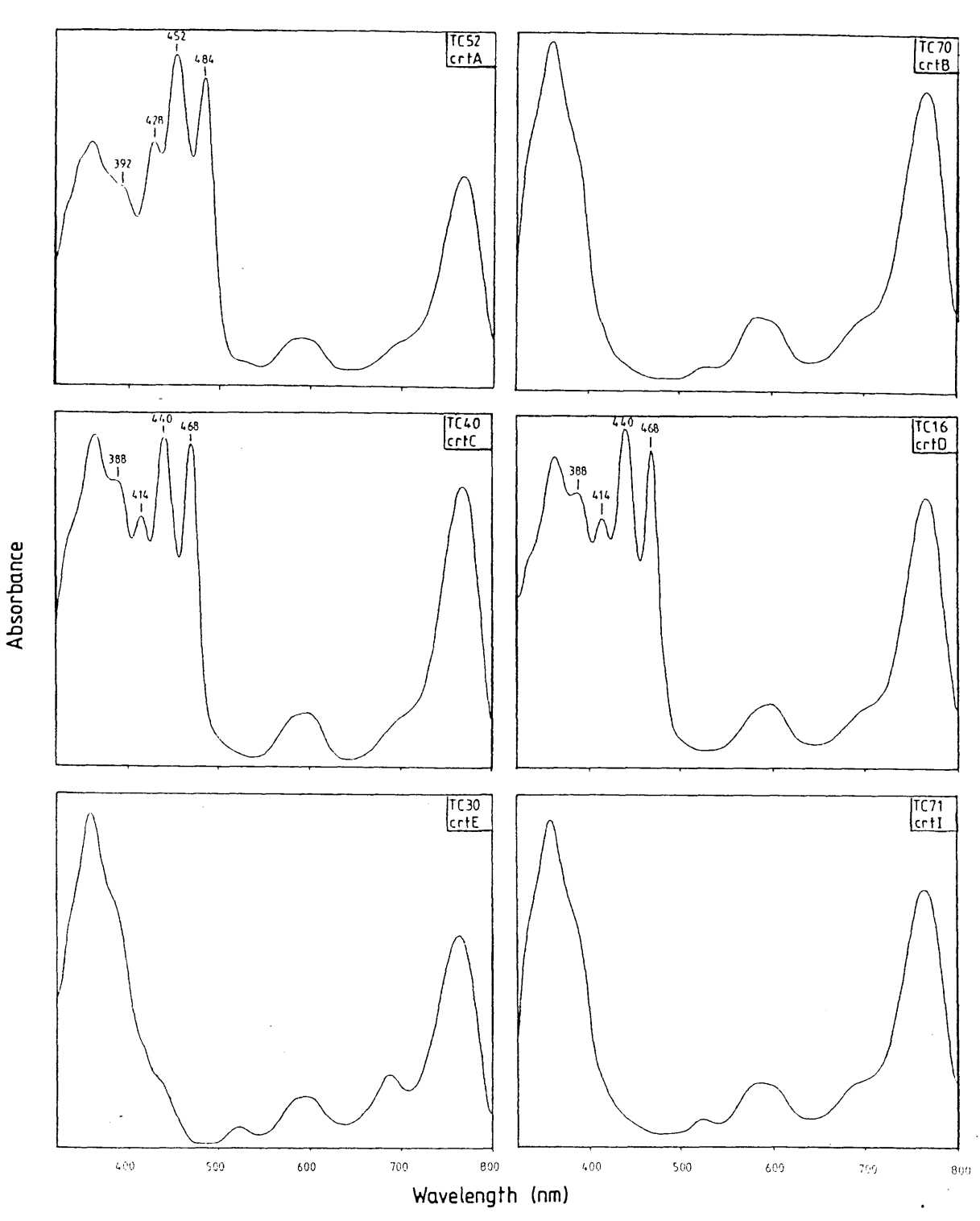


Figure 4.1A

Characteristic absorption spectra of acetone-methanol cell extracts of each type of *Rb. sphaeroides* carotenoid mutant. In each case the vertical axis is equivalent to 1 absorbance unit full scale. crtB, crtE and crtI mutants show a blue green phenotype, the phytoene component of crtI can only be distinguished using HPLC or TLC techniques. crtD and crtC mutants are green carotenoid mutants which have similar absorption profiles and can only be distinguished using HPLC or TLC techniques. Absorption maxima are labelled.

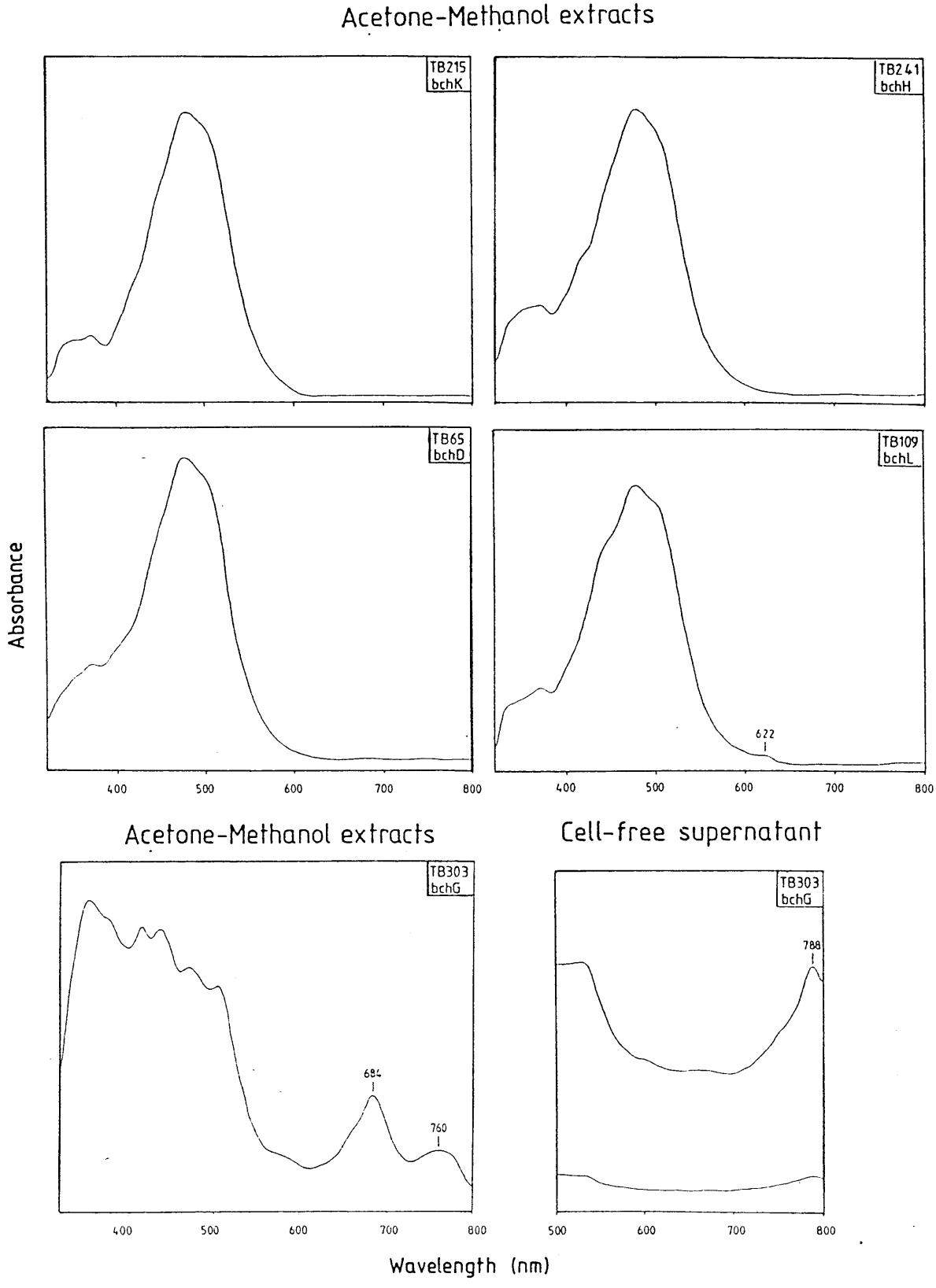


Figure 4.1B

Characteristic absorption spectra of acetone-methanol cell exctracts for each bchl mutant not previously described in chapter 3 (figure 3.1). The vertical axis in each case is equivalent to 1 absorbance unit full scale, except in the second scan of bchG cell free supernatent when it is 0.1. bchK, bchH and bchD mutants are similar and show no bchl intermediate is accumulated. The far red absorption maxima of bchl intermediates are labelled. Apart from bchG the other bchl mutants had featureless cell free supernatent absorption specta. The bchG Tn5 mutant (TB303) was isolated by M. Chaudri.

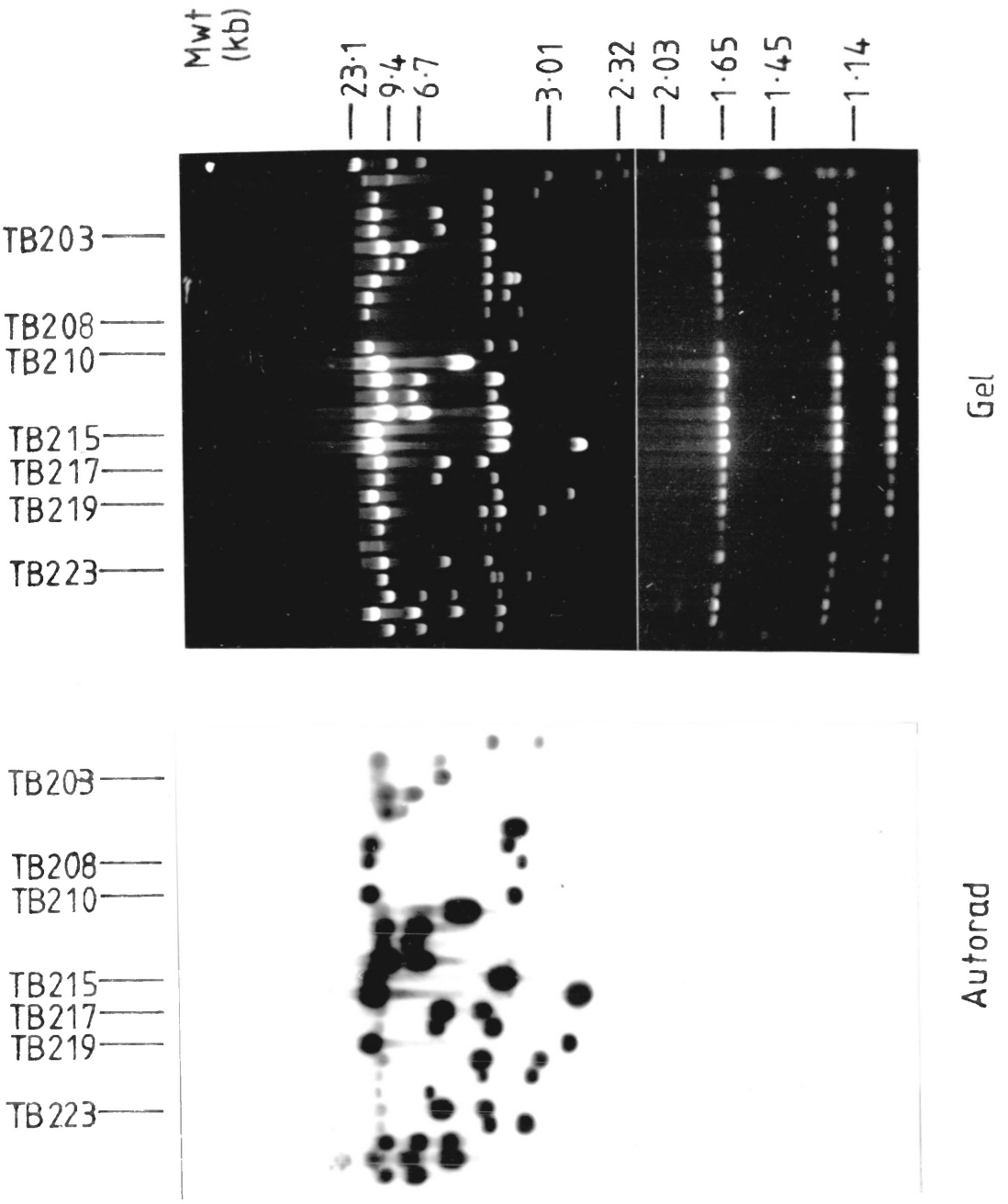


Figure 4.2A

A 1% agarose gel showing a number of pSCN5-1::Tn5 clones cut with *Bam* HI. The Southern blot of the gel was probed with the 3.5 kb *Hind* III fragment of Tn5. The lanes containing plasmids whose Tn5 insertions are presented in figure 4.3A are labelled. Although all the pSCN5-1::Tn5 plasmids on this gel were transfered to *Rb. sphaeroides* only those labelled produced bchl mutants.

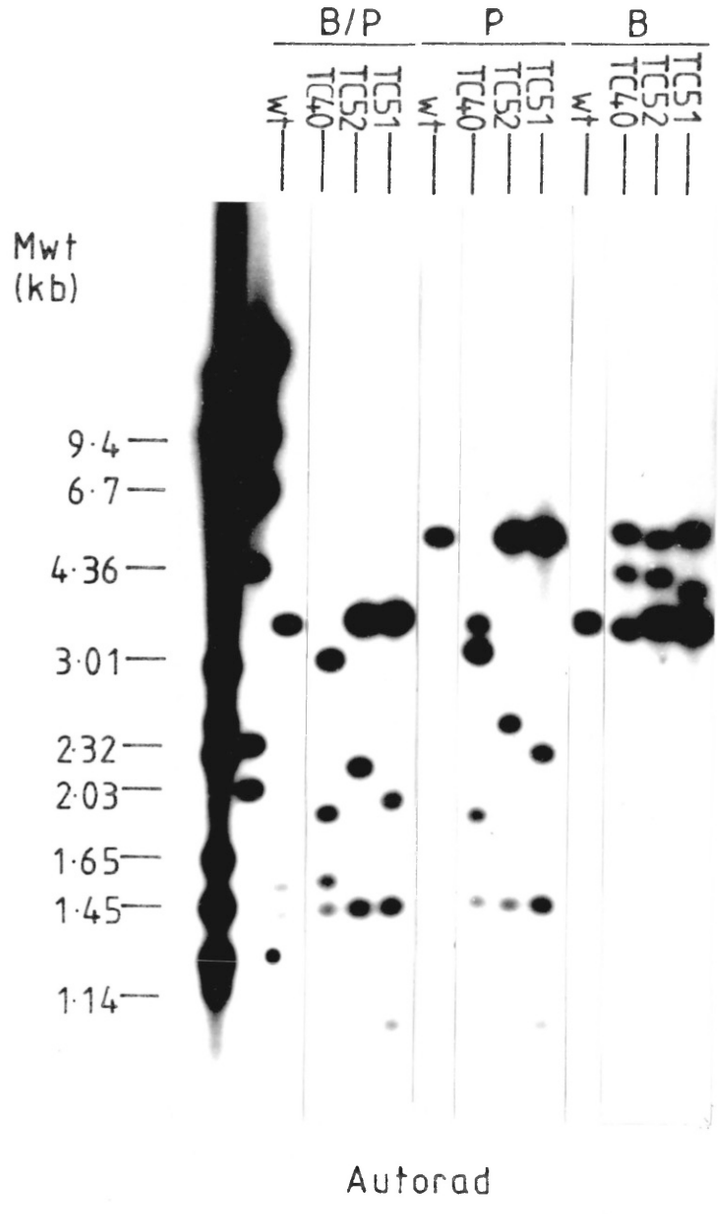


Figure 4.2B

Autoradiograph showing genomic DNA digests from three carotenoid mutants of *Rb. sphaeroides* produced by site directed Tn5 mutagenesis. A mixture of the 3.35 and 3.25 kb *Bam* HI fragments of pSCN22-11 were used as probes. P=*Pst* I, B=*Bam* HI

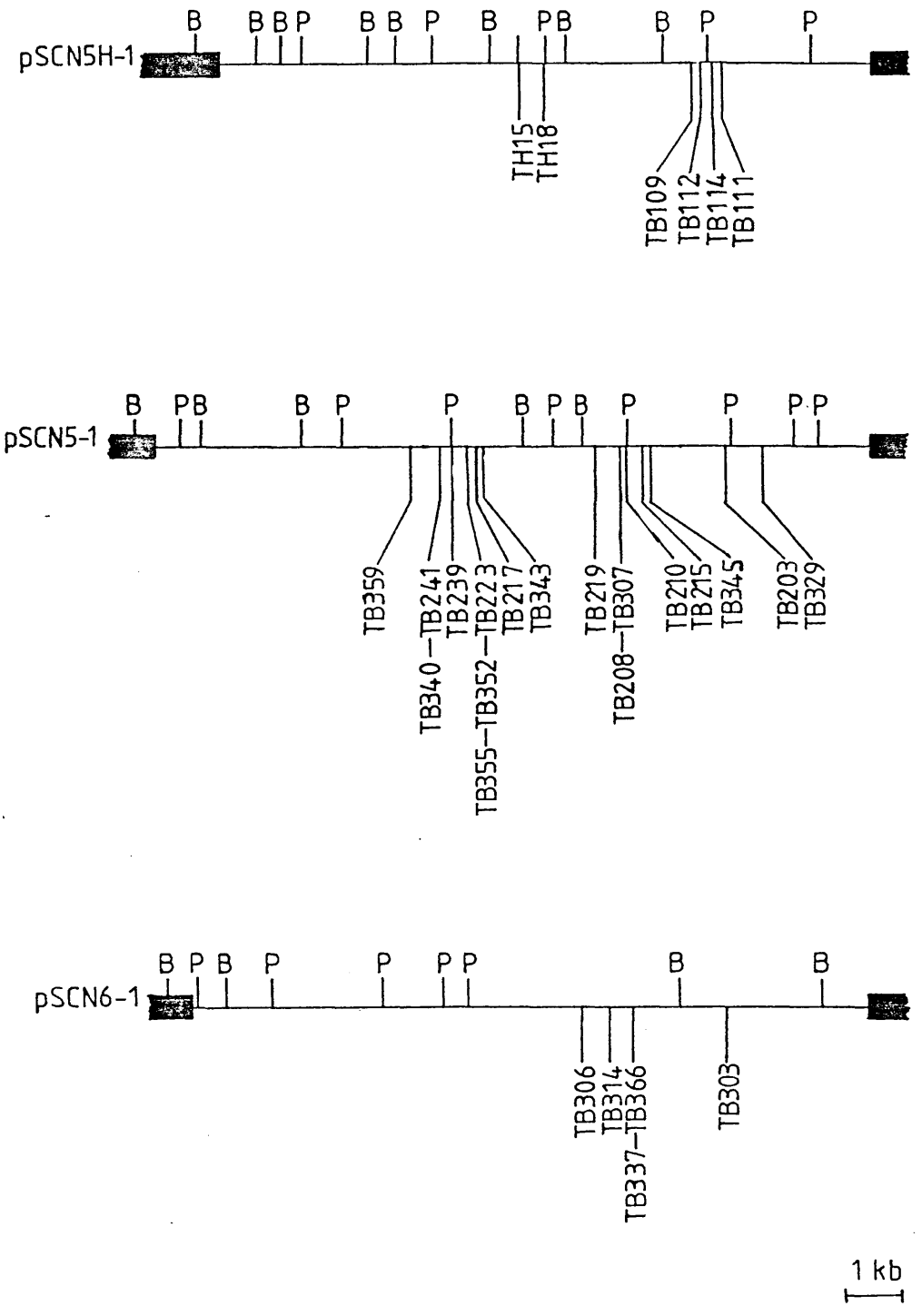


Figure 4.3A

Maps of the DNA inserts of pSUP202 clones pSCN5H-1, pSCN5-1 and pSCN6-1, showing the position of each Tn5 insertion. Table 4.1 lists the mutant phenotype found following transfer to *Rb. sphaeroides* of each of the Tn5 bearing clones. B = *Bam* HI, P = *Pst* I.

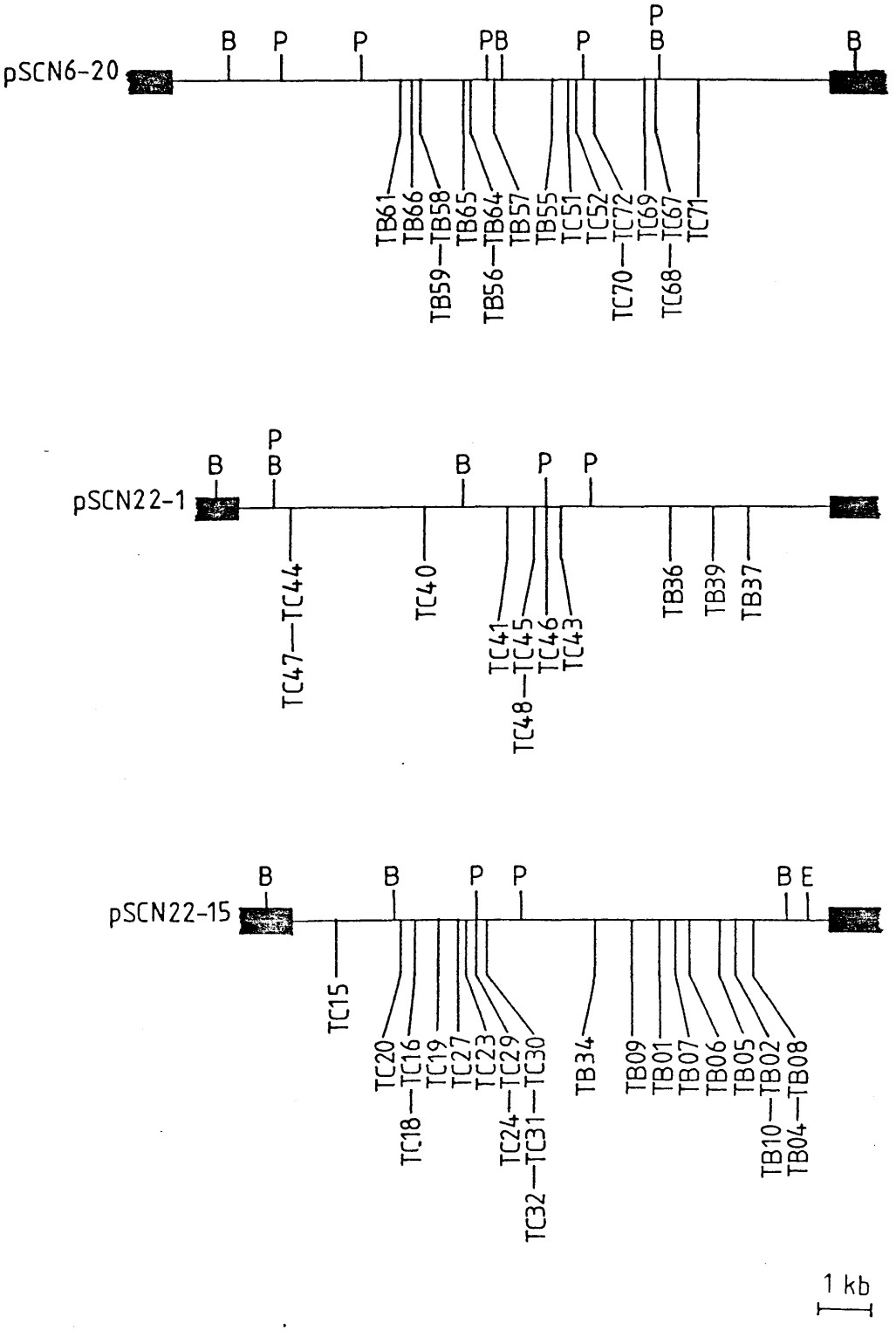


Figure 4.3B

Maps of the DNA inserts of pSUP202 clones pSCN6-20, pSCN22-1 and pSCN22-15 showing the position of each Tn5 insertion. Table 4.1 lists the mutant phenotype found following transfer to *Rb. sphaeroides* of each of the Tn5 bearing clones. B = *Bam* HI, P = *Pst* I.

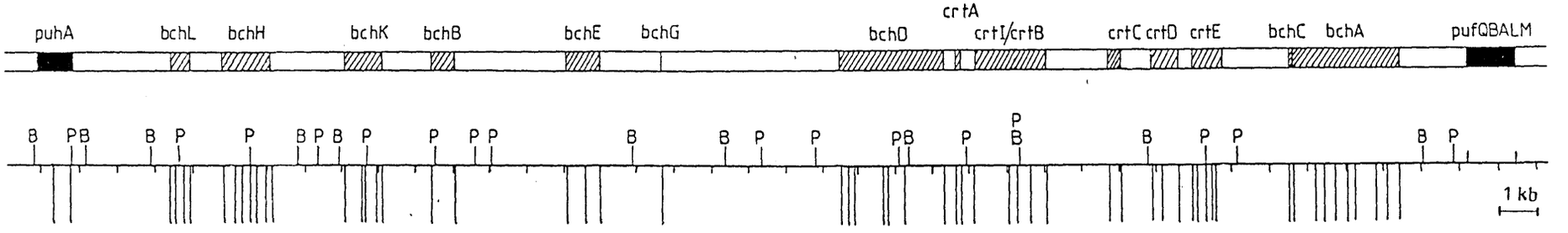


Figure 4.4

Overall map of the *Rb. sphaeroides* photosynthesis gene cluster. Each vertical line represents a Tn5 insertion (in a few cases 2 or 3 identical insertions) derived from data presented in figure 4.3. Each shaded region area indicates a group of Tn5 insertions which show the same crt or bchl mutant phenotype. The block shading indicates areas which have been previously sequenced.

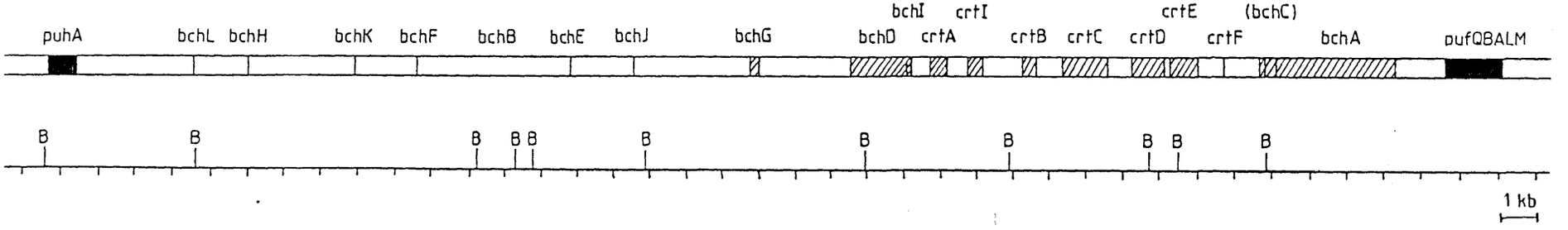


Figure 4.5

The photosynthesis gene cluster of *Rb. capsulatus*. The gene cluster has been redrawn from data presented in a number of papers: Taylor *et al.*, 1983; Biel and Marrs, 1983; Zsebo and Hearst, 1984; Guiliano *et al.*, 1986; Guiliano *et al.*, 1988.

CHAPTER 5

The isolation and characterization of *hem*F encoding a putative anaerobic coproporphyrinogen III oxidase

5.1 Introduction.

The enzyme coproporphyrinogen III oxidase catalyses the oxidative decarboxylation of 2- and 4- propionyl groups of coproporphyrinogen III into vinyl groups to form protoporphyrinogen IX (Chapter 1). Upon induction of photosynthetic membrane in *Rb. sphaeroides* produced by lowering of oxygen concentration from 20% to 1-2%, the flux along the tetrapyrrole pathway increases at least 50 fold. This increase is mainly dedicated to bacteriochlorophyll biosynthesis (Lascelles, 1978), which implies stringent control of Fe^{2+} or Mg^{2+} insertion into protoporphyrin IX. This chelation stage is usually thought to be the branch point at which either haem or bchl synthesis is determined. However, there is evidence that a 'bchl' specific pathway may start at the level of coproporphyrinogen III oxidase where aerobic and anaerobic activities may be specific for haem and bchl, respectively. For example, work by Tait (1969, 1972) suggested that two coproporphyrinogen III oxidase enzymes, one aerobic and one anaerobic, may be present in *Rb. sphaeroides*. Poulson and Polglase (1974) demonstrated differing anaerobic and aerobic coproporphyrinogen III oxidase activities in yeast. In both species the anaerobic enzyme activity required Mg^{2+}, ATP, NAD^+ or $NADP^+$, and methionine or s-adensylmethionine, whereas aerobic activity only required oxygen. Recently, a yeast coproporphyrinogen III oxidase has been purified to homogeneity which was active only when molecular oxygen was used as a electron acceptor and showed no anaerobic activity (Camadro *et al.*, 1986). The *hem*13 gene which encodes this coproporphyrinogen III oxidase in yeast has been sequenced (Zagorec *et al.*, 1988).

This work reports the characterization of a clone which restores wild type phenotype to N1, a *Rb. sphaeroides* mutant which excretes high levels of coproporphyrin III, specifically in low aeration growth conditions. Site directed Tn5 mutagenesis, nucleotide and mRNA analysis have identified a gene tentatively called *hem*F which seems to encode an anaerobic coproporphyrinogen III oxidase.

5.2. RESULTS.

5.2.1. Isolation and biochemical characterization of the N1 mutant of *Rb. sphaeroides.*

N1, a N-methyl-N'-nitro-N-nitro-guanidine (NTG) mutant of *Rb. sphaeroides* was initially isolated by its inability to grow photosynthetically (Dr C.N. Hunter, unpublished). It was quickly discovered that N1 did not make bacteriochlorophyll in semiaerobic culture but instead excreted large amounts of coproporphyrin III, a pink coloured intermediate of haem and bchl biosynthesis. The identity of this tetrapyrrole was established using absorption and nuclear magnetic resonance spectroscopy (Professor O.T.G. Jones, unpublished). Figure 5.1 shows a typical absorption spectra of culture supernatant and acetone : methanol extracts of N1. N1 was found to have wild type levels of succinate cytochrome c reductase and NADH-cytochrome c reductase activity and appears to grow normally in chemoheterotrophic conditions. However, it was found to contain only 20% of the cytochrome c and b in anaerobic conditions compared to the *bch*L mutant O1, although in aerobic conditions concentrations of both cytochromes were similar (I. Lloyd and Professor O.T.G. Jones unpublished). Unlike previously described *Rb. sphaeroides* mutants which excreted coproporphyrin (Lascelles, 1966b; Lessie and Sistrom, 1964) N1 does not require methionine since this amino acid is not a component of $M22^+$ growth medium. Using cell free extracts prepared from semi-anaerobic/dark and anaerobic/light grown N1 and wild type *Rb. sphaeroides*, a number of experiments have been performed to test coproporphyrinogen III oxidase activity which utilised HPLC and TLC techniques. On incubation with porphobilinogen wild type cell-free extract produced uroporphyrin, coproporphyrin III and protoporphyrin IX. However, Nl cell-free extract produced only uroporphyrin, coproporphyrin III and a peak attributed to the 3 - carboxyl intermediate harderoporphyrin. Incubation of wild type cell-free extract with coproporphyrinogen III produced a small amount of protoporphyrin IX which coincided with a decrease in the coproporphyrinogen III peak. In a similar experiment N1 cell-free extract showed no change in level of coproporphyrinogen and no protoporphyrin could be detected. It was concluded that the mutant Nl contained negligible levels of coproporphyrinogen III oxidase activity (R. Jones and Dr P.M. Jordan, unpublished).

5.2.2. Isolation of a clone which restores Nl to wild type phenotype.

Initially, a clone was isolated from the pNH2 bank of *Rb. sphaeroides* genomic DNA using a procedure already described in section 3.2.4. The clone pSC124, which restored Nl to wild type phenotype, was mapped using restriction endonucleases *Pst* I, *Eco* RI, *Bam* HI, and *Hind* III (figure 5.2), and later *Sma* I, *Sst* I and *Acc* I (shown in figure 5.5B).

5.2.3. Location of the N1 gene using random and site directed Tn5 mutagenesis.

Initially two randomly generated Tn5 mutants of *Rb. sphaeroides* which displayed a Nl-like phenotype were isolated using the method described in section 2.6.5.1. Genomic DNA was prepared from both Nl-like Tn5 mutants and using genomic hybridisation techniques the position of each Tn5 insert was mapped using pSC124 DNA as a probe. In order to examine the location of the gene using site directed Tn5 mutagenesis, three clones pSCN1-1, pSCN1-2 and pSCN1-3 were isolated from the bank of *Rb. sphaeroides* DNA in the 'suicide' vector pSUP202, using the 1.05 kb *Eco* RI fragment of pSC124 as a probe. On transfer from *E. coli* all three clones restored N1 to wild type phenotype. pSCN1-1 was mapped using restriction endonucleases *Eco* RI, *Hind* III, *Pst* I and *Bam* HI (Figure 5.3). Three more Tn5 mutants showing an N1-like phenotype were isolated using pSCN1-1 and the site-directed mutagenesis technique (section 4.2). Figure 5.4 shows that the three Tn5 insertions which produce a N1-like phenotype are in the 1.05 kb *Eco* RI fragment. No other non-photosynthetic phenotype was detected for the Tn5 insertions in other regions of the pSCN1-1 insert. Figure 5.5A shows the restriction map of pSCN1-1 and the positions of each Tn5 insertion.

5.2.4. Nucleotide sequencing.

A number of clones were constructed in the phage vector M13 mp18/19 which covered a 2.7 kb region of pSC124 and which included the sites of all five Tn5 insertions which produced a N1-like phenotype. Figure 5.5B shows the extent and direction of the sequencing using the dideoxy nucleotide method (section 2.5). The nucleotide sequence obtained (figure 5.6) was analysed using the Wisconsin package. Using the CODONPREFERENCE programme an open reading frame of 1269 bp long was identified (Figure 5.7). Of the three possible methionine residues (306, 747 and 792 bp) the second two methionine residues are preceded by possible Shine-Delgarno sequences (marked on figure 5.6). Translation of the open reading frame from the methionine residue at 747 bp gives a polypeptide of 250 amino acid residues

in length with a calculated molecular weight of 28,250. An inverted repeat of 11 bp was identified, which could function as a transcription terminator; this was found 180 bp from the end of the open reading frame (indicated on figure 5.6). Furthermore, a search with the oxygen regulated *puf*1 promoter of the *puf* operon of both *Rb. sphaeroides* and *Rb. capsulatus* revealed a sequence between 288 and 300 bp on figure 5.6 which showed a significant degree of homology (Figure 5.8).

5.2.5. Northern analysis of the mRNA transcript of the *hem*F open reading frame.

Total RNA was isolated at time intervals 0, 0.5, 1, 2, and 4 hours (section 2.4.2.) from *Rb. sphaeroides* cells undergoing pigment synthesis induced by lowering the oxygen concentration (section 2.3.3.). Figure 5.9 shows the increase in pigment-protein complexes in wild type *Rb. sphaeroides* over this time period. A number of Northern blots were prepared from total RNA from *Rb. sphaeroides* (section 2.4.13) and probed with a number of single-stranded probes derived from M13 clones (section 2.5.4). Figure 5.10A shows the extent and direction of each M13 clone used. Three of the single stranded M13 probes hybridize to a 1.3 kb transcript (Figure 5.10B) confirming that the coding strand identified by codon preference analysis was transcribed. The three autoradiograms were scanned on a Shimadzu CS-930 dual-wavelength scanner and showed a 2-fold increase in the level of the 1.3 kb transcript in the first 30 minutes following lowering of oxygen concentration. This result is in broad agreement with the kinetics of mRNA induction observed when pSC124 was used as a probe and parallels the rise observed when *bch* transcripts are studied (Coomber *et al.*, 1987; Hunter and Coomber, 1988).

5.2.6. Nuclease S1 analysis of the 1.3 kb *hem*F transcript

The 5' end of the 1.3 kb mRNA transcript was mapped using the M13 clone N1-82A (mp19 carrying the 570 bp *Eco* RI - *Sma* I fragment), and the method described in section 2.9. Figure 5.11 shows a single band around 420 bp in size. This result places the 5' end of the mRNA transcript in the position predicted from homology with the *puf*1 promoter (shown on figure 5.6).

5.2.7. Comparison of the *hem*F open reading frame with the *hem*13 gene of yeast.

Recently the *hem*13 gene of yeast which encodes coproporphyrinogen III oxidase has been sequenced (Zagorec *et al.*, 1988). The amino acid sequence of the *hem*13 gene was compared to that obtained for the *hem*F open reading frame (Figure 5.12). The two polypeptides showed a percentage similarity of 44.7 with 14 gaps.

5.2.8. Hydropathy data of the *hem*F opening reading frame

The amino acid sequence of the *hem*F open reading frame was analysed using the PEPPLOT programme of the Wisconsin package (figure 5.13). The hydropathy data indicates that this open reading frame encodes a polypeptide which has a hydrophylic character consistent with a cytosolic enzyme.

5.3. DISCUSSION.

The sequence data indicated an open reading frame of 1269 bp which exhibited the use of codons most frequently utilised in all the other *Rb. sphaeroides* genes that have been sequenced (*puf*QBALM, *puc*AB, *puh*A and *cyc*A). No other open reading frame was as long or showed typical *Rb. sphaeroides* codon usage to the same degree. An area which showed similarity to the *puf*1 promoter of the *puf* operon was identified at the start of the open reading frame. Furthermore, a stem-loop structure which could function as a transcription terminator was found 180 bp from the end of the open reading frame. This data would indicate a mRNA species of around 1.34 kb, which agreed with the 1.3 kb mRNA transcript found on Northern analysis. Nuclease S1 mapping of the 1.3 kb mRNA showed that the 5' end of the transcript agreed with the transcription start site predicted by homology to the *puf*1 promoter. It therefore seems likely that the promoter region of this gene has been identified. The 1.3 kb transcript increased 2-fold when oxygen concentration was lowered. Transcript levels encoding *bch* genes in *Rb. sphaeroides* also increase on the same timescale with these kinetics (Coomber *et al.*, 1987; Hunter and Coomber, 1988). The homology of the promoter region of the *hem*F with *puf*1 promoter may be indicative of control by similar oxygen-regulated mechanisms.

As the methionine residue at 306 bp falls before the mapped 5' transcript start and lacks a convincing Shine-Delgarno sequence it seems likely that the methionine residue at 747 bp is the first residue of the polypeptide. Although a gap of 439 bp exists between the *hem*F promoter and this first methionine, it should be noted that similar gaps are found in both *Rb. capsulatus* and *Rb. sphaeroides puf* operons. The

space between the *puf*1 promoter and the first methionine of pufQ is 315 bp in *Rb. sphaeroides* (Ashby, 1988), and 365 bp in *Rb. capsulatus* (Bauer *et al.*, 1988). It is possible that this gap is a common feature of oxygen regulated promoters of genes involved in photosynthesis in *Rhodospirillacae*.

Comparison with the 328 amino acid residues of the gene product of the *hem*13 gene of yeast showed a percentage similarity of only 44.7 with 14 gaps. *Hem*13 has been shown to encode coproporphyrinogen III oxidase, a homodimer with a subunit molecular wt of 35,000, which is found in the cytosol of yeast (Camadro *et al.*, 1986; Zagorec *et al.*, 1988). The hydropathy data indicates that the *hem*F open reading frame encodes a fairly hydrophilic polypeptide consistent with the observation in yeast that coproporphyrinogen III oxidase is a cytosolic enzyme. Although the homology at amino acid is not strong, level this does not necessarily indicate that the 1269 bp open reading frame does not encode coproporphyrinogen III oxidase.

Biochemical data suggests that lesions located within the *hem*F open reading frame are consistent with the loss of an anaerobic coproporphyrinogen III oxidase dedicated to bacteriochlorophyll biosynthesis. Although coproporphyrin III excretion is a feature of methionine requiring mutants of *Rb. sphaeroides* (Lascelles, 1966b, Lessie and Sistrom, 1964), an observation that is consistent with the 5-adenosyl methionine requirements of anaerobic coproporphyrinogen oxidase activity (Tait, 1969, 1972), N1-like mutants do not require methionine for growth. Previously enzymes involved in the biosynthesis of protoporphyrin IX from 5-aminolevulinic acid synthase to protoporphyrinogen oxidase were assumed to be shared by both the haem and bacteriochlorophyll pathways in photosynthetic organisms. The work presented here together with the observation of two coproporphyrinogen III oxidase activities, aerobic and anaerobic (Tait 1969, 1972) and more recently the residual 10% ALA synthase activity of a *hem*A mutant of *Rb. capsulatus* (Wright *et al.*, 1988) suggest a new model. This would propose that both haem and bacteriochlorophyll biosynthesis proceed along distinct pathways, possibly in separate areas of the cell and subject to differing regulatory mechanisms, and explain why complete disruption of the gene reported here does not result in the fatal absence of haem biosynthesis.

The simplest explanation of the observation that the presence of pSCN1-1 in *E. coli* produces a 2-fold increase in coproporphyrinogen III oxidase activity (Dr P.M. Jordan and R. Jones, unpublished) is that the plasmid carries the gene for the enzyme. It is therefore proposed that the open reading frame identified in this work should be tentatively called *hem*F. However further work needs to be undertaken to prove that the *hem*F gene product has coproporphyrinogen III oxidase activity. Over expression of the *Rb. sphaeroides hem*F gene product in *E. coli*, purification and amino terminal sequence of the polypeptide, followed by detailed enzymatic analysis will be

necessary to show that *hem*F encodes for an anaerobic coproporphyrinogen III oxidase.

Recently, it has been shown that the plasmid pSCN1-3 bears both the *puc*AB operon encoding LHI polypeptides and *hem*F (M. Chaudri, S.A. Coomber and C.N. Hunter, unpublished). It has been shown that these two loci map 2.8 kb apart (A. Rowell, M. Chaudri, S.A. Coomber and C.N. Hunter, unpublished). It should be noted that the *puc*AB operon does not lie within the main 46 kb photosynthetic cluster of *Rb. sphaeroides*. The linkage of *hem*F with a gene involved in the photosynthetic apparatus lends credence to the suggestion that *hem*F encodes an anaerobic coproporphyrinogen III oxidase dedicated to bacteriochlorophyll biosynthesis.

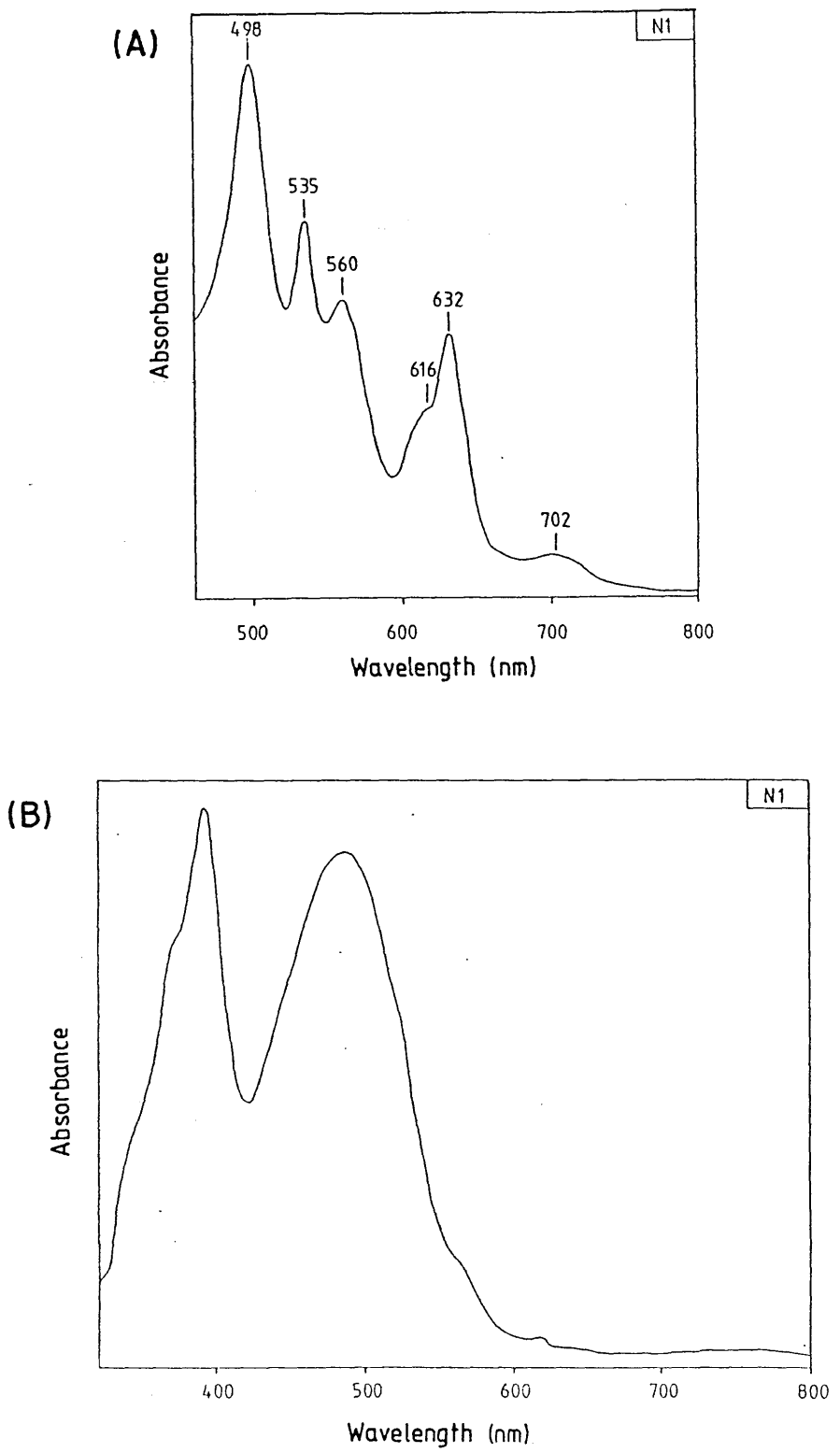


Figure 5.1

The absorption spectra from

(A) the cell free supernatant

(B) acetone-methanol cell extract

of the *Rb. sphaeroides* mutant N1. The vertical axis is equivalent to 1 absorbance unit full scale. The absorbance maxima are marked.

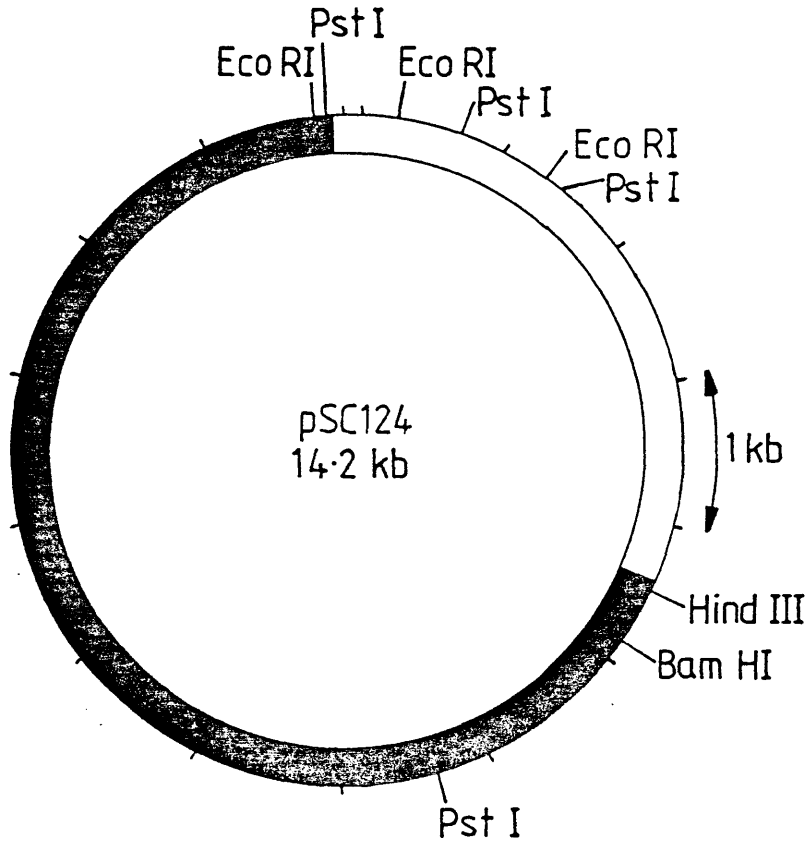


Figure 5.2

The restriction map of pSC124 showing *Eco* RI, *Bam* HI, *Pst* I and *Hind* III sites. The vector pNH2 is shaded.

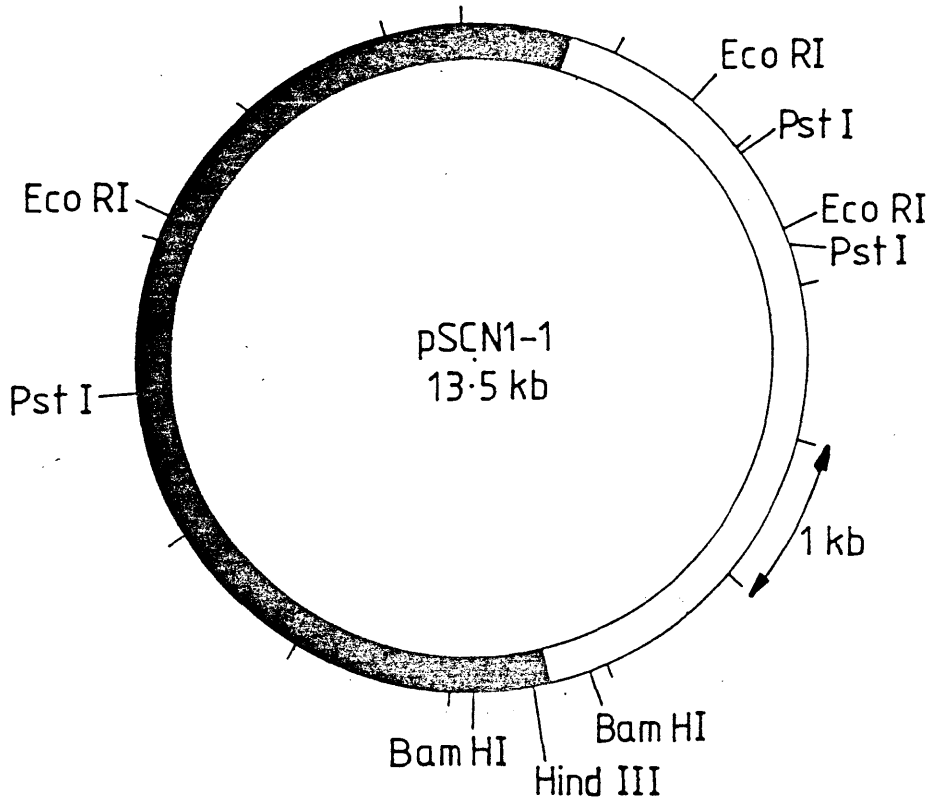


Figure 5.3

The restriction map of pSCN1-1 showing *Eco* RI, *Bam* HI, *Pst* I and *Hind* III sites. The vector pSUP202 is shaded.

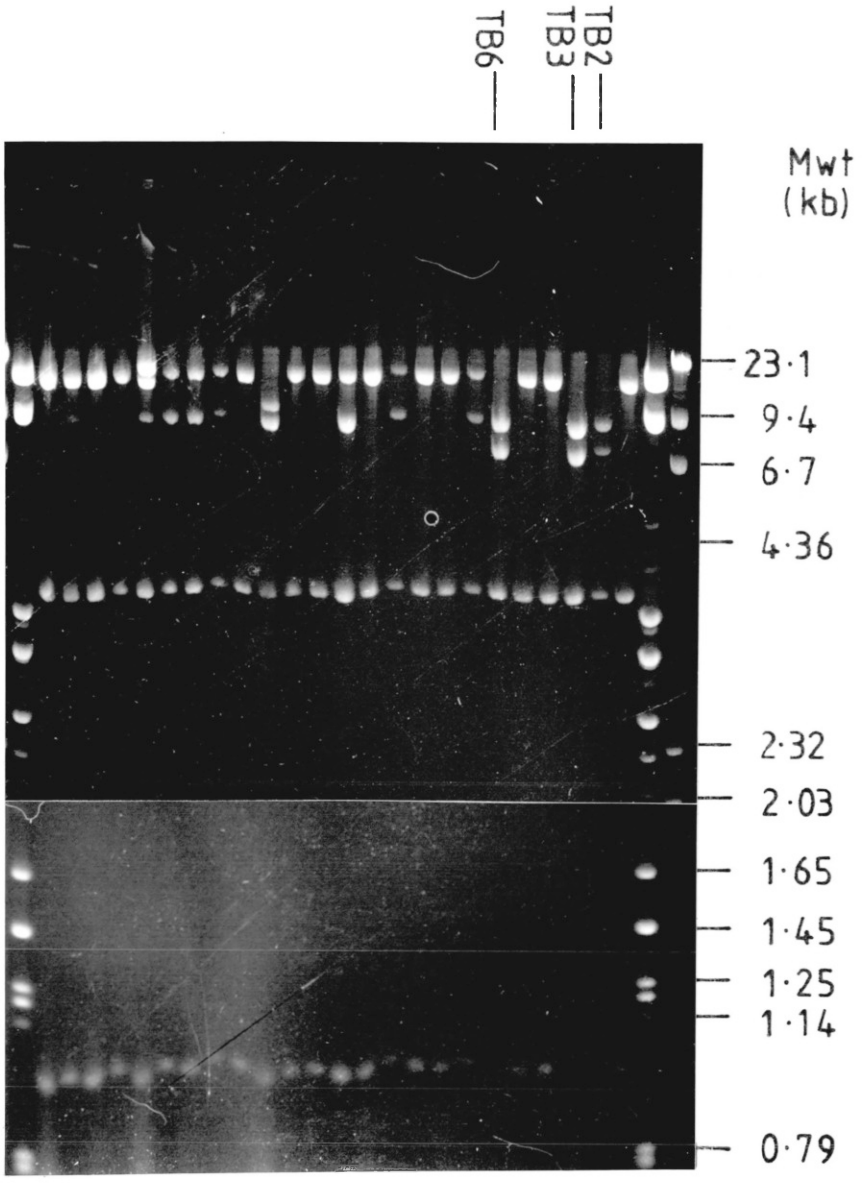


Figure 5.4

A 1% agarose gel showing a number of pSCN1-1::Tn5 clones cut with *Eco* RI. TB2, TB3 and TB6 produced a N1-like phenotype on transfer to *Rb. sphaeroides* and clearly show Tn5 has inserted into the 1.05 kb *Eco* RI fragment. Although all the pSCN1-1::Tn5 clones shown on this gel were transfered into *Rb. sphaeroides* only TB2, TB3 and TB6 produced a non-photosynthetic mutant phenotype.

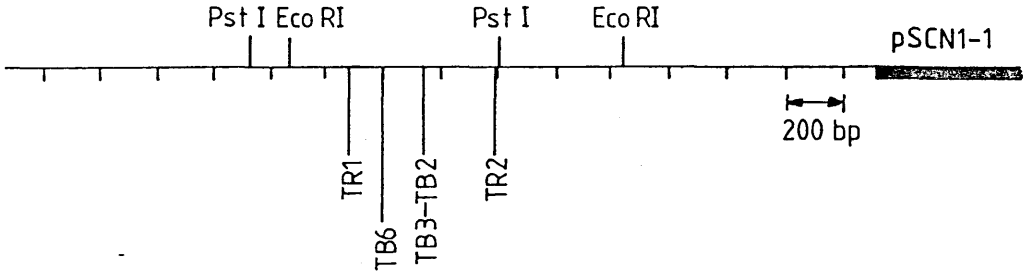


Figure 5.5A

Map of part of pSCN1-1 showing the positions of the three site directed Tn5 insertions which produced a N1-like phenotype on transfer to *Rb. sphaeroides*. The positions of the randomly generated Tn5 insertions which also show a N1-like phenotype are also marked (TR1 and TR2).

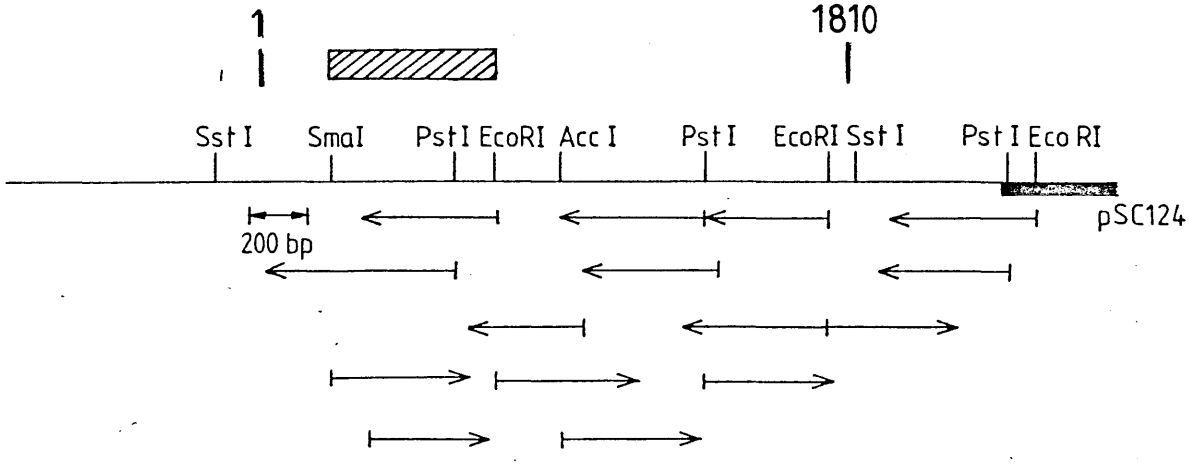


Figure 5.5B

Map of part of pSC124 showing the direction and extent of DNA sequencing. 1 and 1810 indicate the position of sequence shown in figure 5.6. The shaded box indicates the *Eco* RI - *Sma* I fragment used for S1 nuclease analysis.

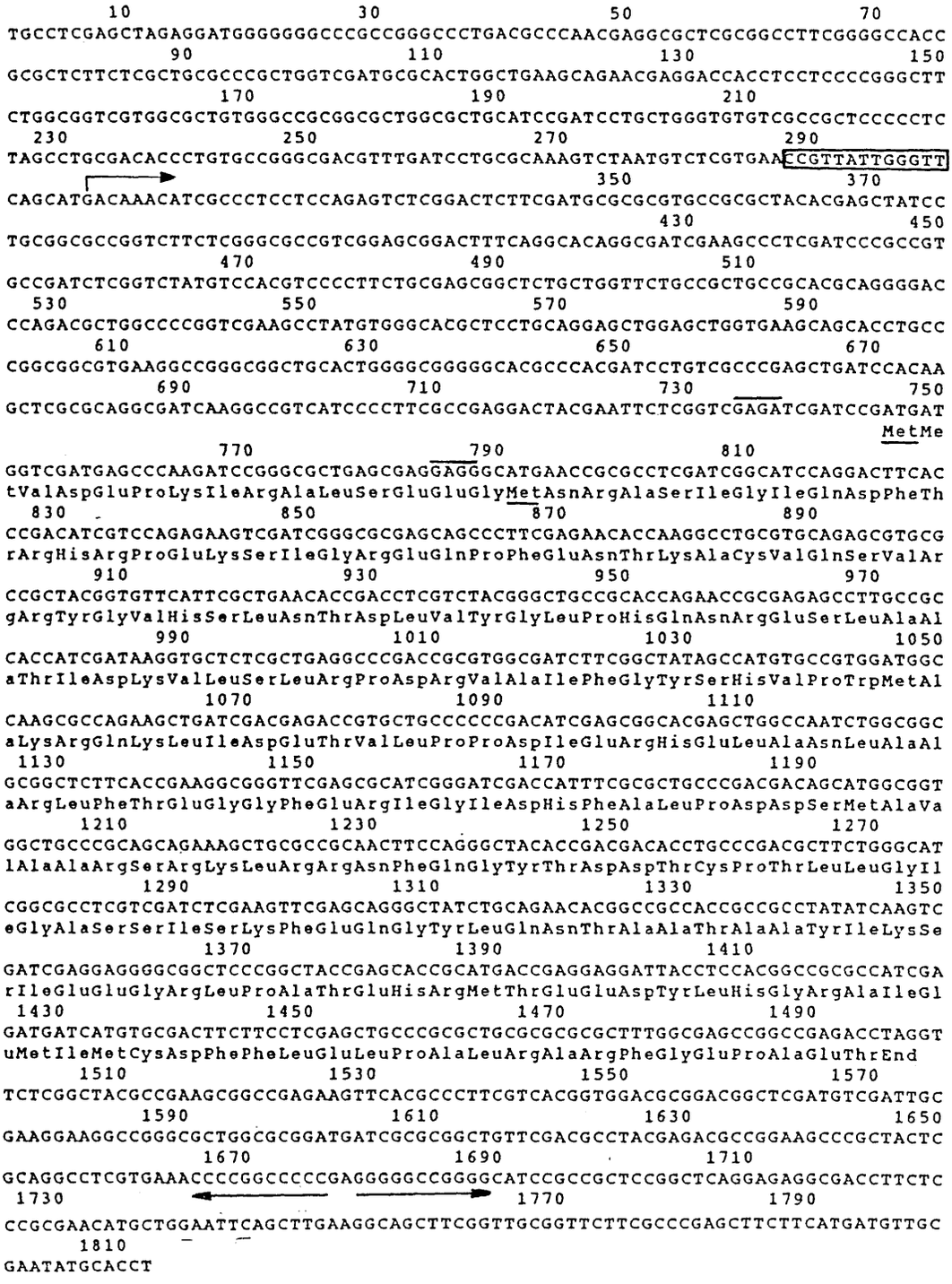


Figure 5.6

Nucleotide sequence and deduced amino acid sequence of the putative *hem*F gene. The two possible initiating methionines are underlined along with possible Shine-Dalgarno sequences. The stem-loop structure which could function as a transcription terminator is marked by arrows. The start of the 5' end of the mRNA is marked ⌐→. The region which shows strong homology to the oxygen regulated *puf*1 promoter of both the *Rb. sphaeroides* and *Rb. capsulatus puf* operons is boxed.

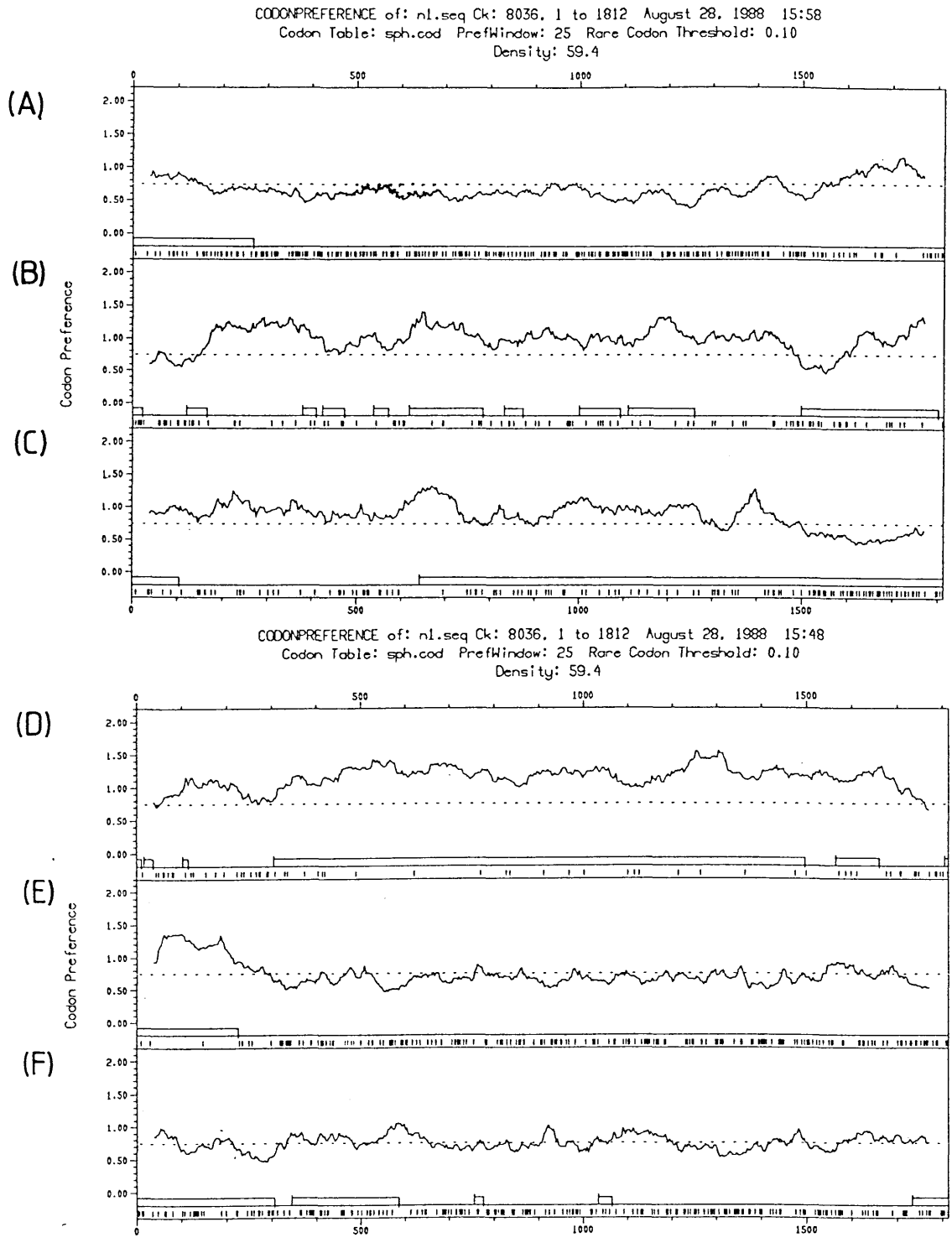


Figure 5.7

The codon preference plot of the nucleotide sequence shown in figure 5.6 in all six possible reading frames. (D) has a open reading frame running from 300 to 1500 bp which shows the good use of codons most frequently found in other *Rb. sphaeroides* genes. The amino acid sequence from part of this open reading frame is shown in figure 5.6.

```
-50      -40      -30      -20      -10      +1

CCCGGGTGCGGCGATCCGGCGCGCTACCGGAACGCCCGTTATGGGATATGCAGG   Rb. sphaeroides puf1
*** ** ********** ***** ** ** *** * ****** **    **
CCCCGGCGCGGCGATCCGCCGCGCGACGGGCACCCCCCTTCATGGGTTACATGGG  Rb. capsulatus puf1
   *  * ***   * *   * *   *  ** **  ******      *
GTTTGATCCTGCGCAAAGTCTAATGTCTCGTGAACCGTTATTGGGTTCAGCATG   Rb. sphaeroides hemF
   * * * ***   * *     * *    **  *  **** *  *** *
CCCGGGTGCGGCGATCCGGCGCGCTACCGGAACGCCCGTTATGGGATATGCAGG   Rb. sphaeroides puf1
```

Figure 5.8

A region upstream of the initiation codon of the putative *hem*F gene product shows strong homology to the oxygen regulated *puf*1 promoter of both *Rb. sphaeroides* and *Rb. capsulatus*. Identical bases are marked with an asterisk.

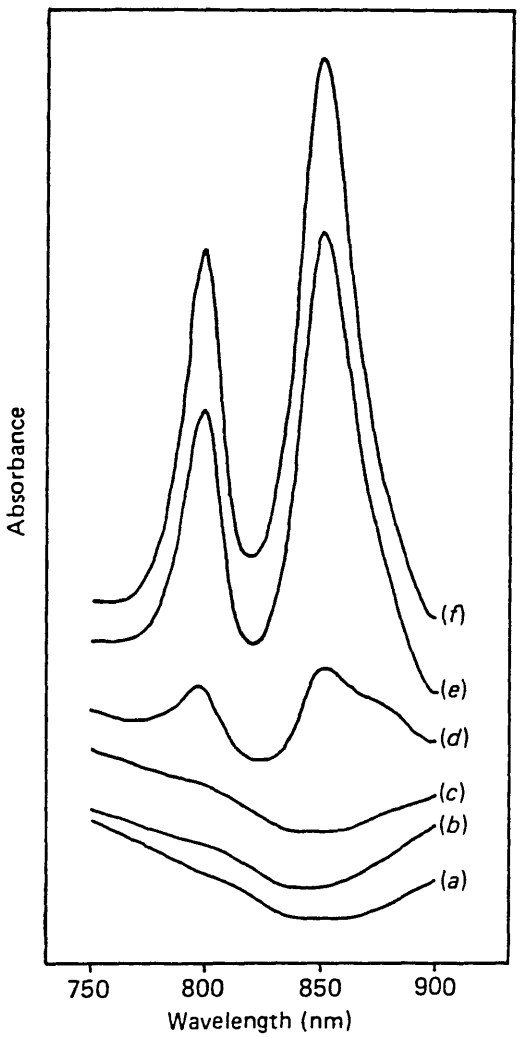


Figure 5.9

Absorption spectra of whole cells in glycerol showing induction of the photosynthetic apparatus of *Rb. sphaeroides*. (a)-(f) represent samples removed from the culture after 0, 0.5, 1, 2, 4, and 6 hours of induction (section 2.3.3). The same wet weight of cells was used for each time point.

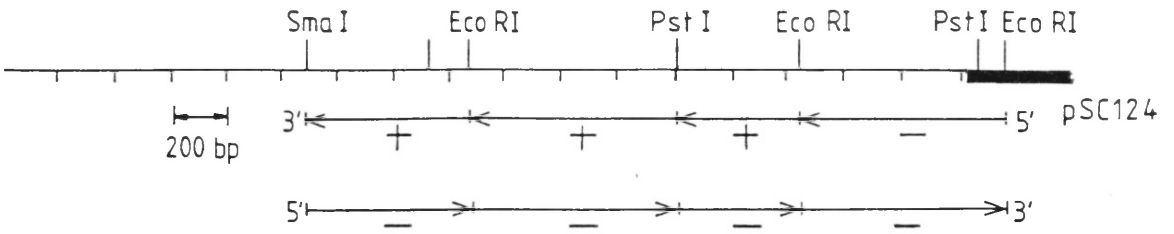


Figure 5.10A

A map of pSC124 showing the various M13 derived single stranded probes used in the Northern analysis of *hem*F of *Rb. sphaeroides*. + indicates those probes which showed a 1.3 kb mRNA transcript (see figure 5.10B for typical blot). - indicates that no mRNA transcript was found.

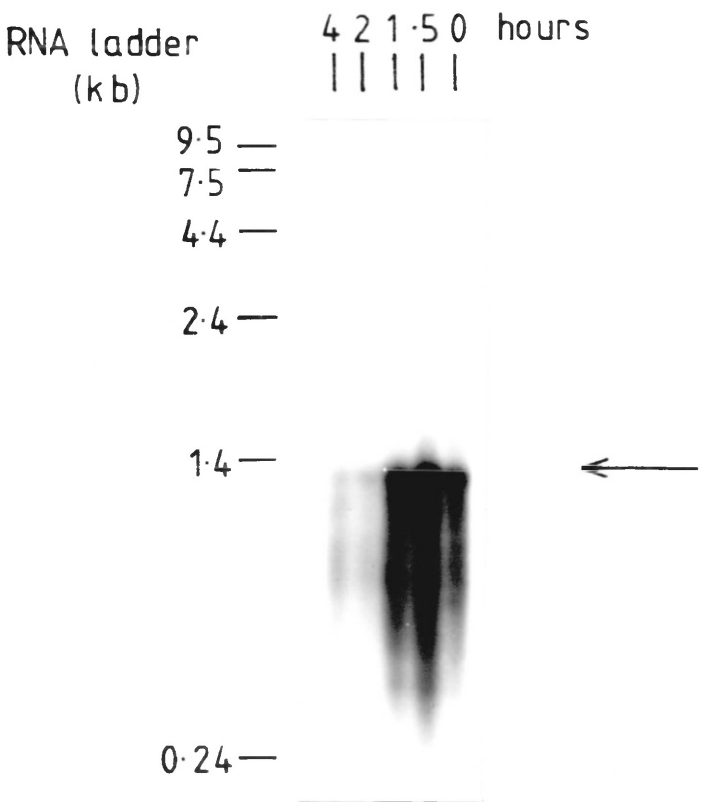


Figure 5.10B

A typical Northern blot showing the 1.3 kb mRNA transcript of *hem*F (probed with one of + M13 clones shown in figure 5.10A). Total RNA was extracted from *Rb. sphaeroides* cells at 0, 0.5, 1, 2 and 4 hours after semi- anaerobic induction (section 2.3.3) and 5μg was loaded in each lane.

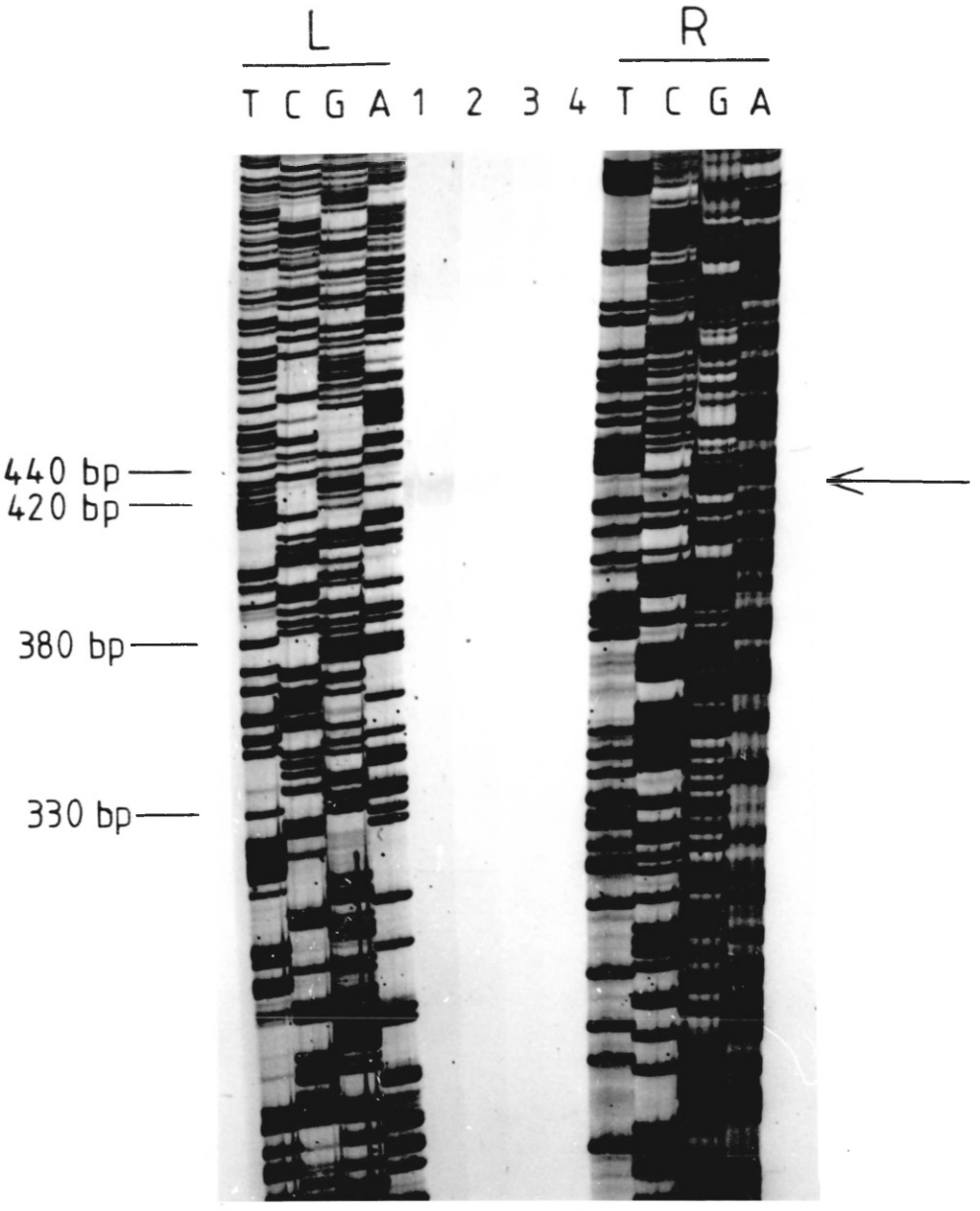


Figure 5.11

S1 protection analysis. *Rb. sphaeroides* RNA extracted 1 hour after the start of anaerobic induction was used. The *Eco* RI - *Sma* I fragment shaded on figure 5.5B cloned in M13mp19 (mp19-82A) was used as the source of ssDNA probe. R=mp19-82A and L=M13mp18. The arrow points to the band. 1-4 represent a 2-fold dilution series of the product of nuclease S1 digestion. The 5' end is indicated on figure 5.6.

```
        Gap Weight:  2.000      Average Match:  0.540
     Length Weight:  0.300   Average Mismatch: -0.396

           Quality:   87.6             Length:    302
             Ratio:  0.352               Gaps:     14
Percent Similarity: 44.758

N1.Pep x Hem13.Pep        September 12, 1988  20:43  ..

      .         .         .         .         .
  2 MVDEPKIRALSEEGMNRASIGIQ..DFTRHRPEKSIGREQPFENTKACVQ 49
    |     | **          *||  *  | |||     ||  | *   |*
 10 LPIRQQMEALIRRKQAEITQGLESIDTVKFHADTWTRGNDGGGGTSMVIQ 59
      .         .         .         .         .
 50 SVRRYGVHSLNTDLVYG.LPHQNRESLAATIDKVLSLRPDRVAIFGYSH. 97
       ||  ||*  |***** *       | *   * * * *  *    |
 60 DGTTFEKGGVNVSVVYGQLSPAAVSAMKAD.HKNLRLPEDPKTGLPVTDG 108
      .         .         .         .         .
 98 VPWMAKRQKLIDETVLPPD.........IE.RHELANLAARLFTEGG... 134
    * ||*    ||   * *           |* ||| |    ||* |*|
109 VKFFACGLSMVIHPVNPHAPTTHLNYRYFETWNQDGTPQTWWFGGGADLT 158
      .         .         .         .         .
135 ....FERIG..IDHF...ALPDDSMAVAARSRKL.........RRN.... 162
       |*  *  | ||   **     *! |* |*|          *||
159 PSYLYEEDGQLFHQLHKDALDKHDTALYPRFKKWCDEYFYITHRKETRGI 208
      .         .         .         .         .
163 ....FQGYTDDTCPTLLGIGASSISKFEQGYLQNTAATAAYIKSIEEGRL 208
       *||* |      |* |    ||  *  |**               **  |
209 GGIFFDDYDERDPQEILNMVEDCFDAFLPSYLTIVKRRKDMPYTKEEQQW 258
      .         .         .         .         .
209 PATEH.RMTEEDYLHGRAIEMIMC.......DFFLELPALRARF..GEPA 248
     *  | *  * | | |*| || |          ||| **    ||    **
259 QAIRRGRYVEFNLIYDRGTQFGLRTPGSRVESILMSLPEHASWLYNHHPA 308

249 ET 250

309 PG 310
```

Figure 5.12

A comparision of *hem*13 and the putative *hem*F gene products using BESTFIT. Identical amino acid residues are indicated by astericks and conservative changes by dashed lines. The numbers refer to the position of the amino acid residues in the *hem*F gene product (this chapter) or the *hem*13 gene product (Zagorec et al., 1988).

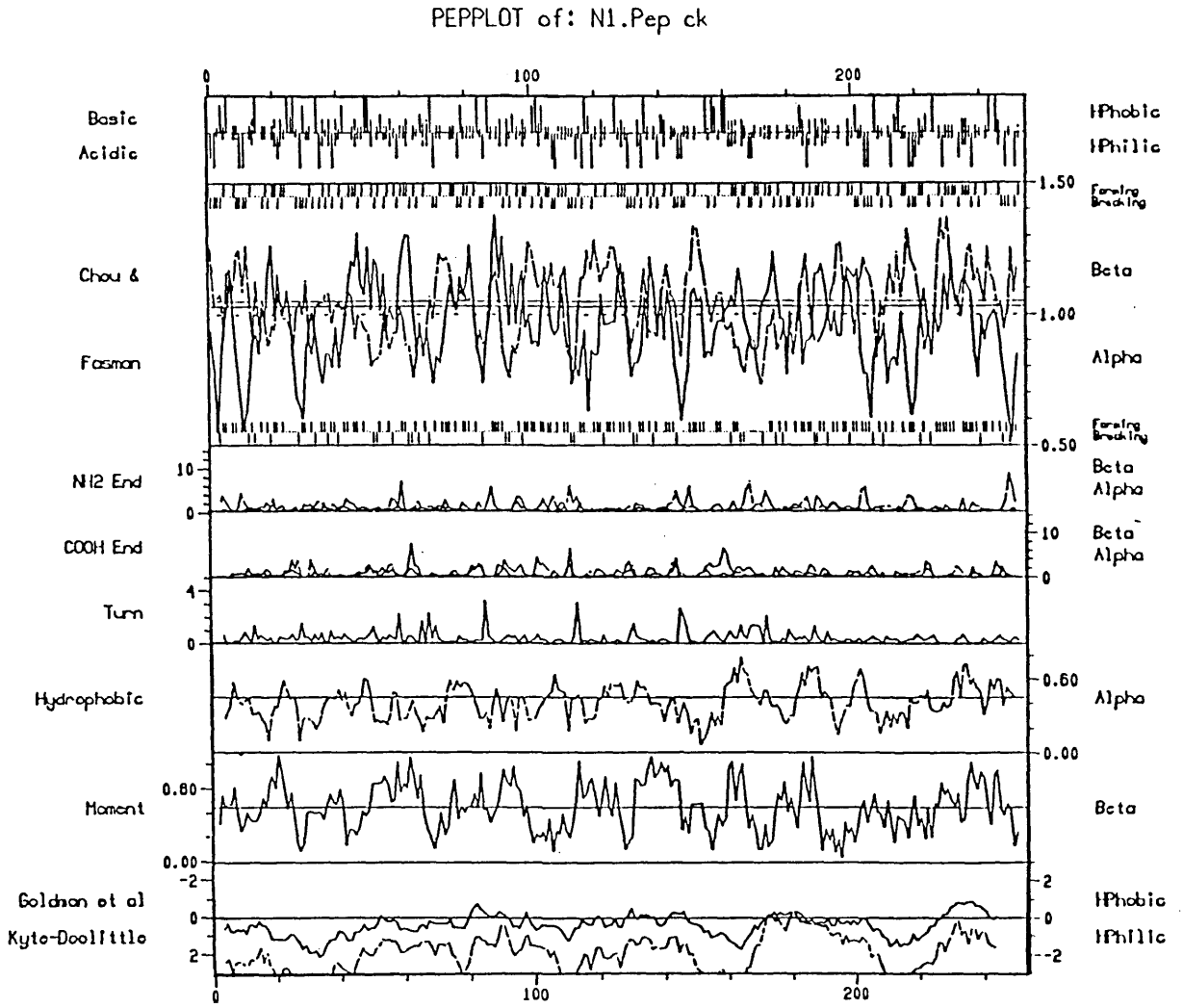


Figure 5.13

The PEPPLOT of the *hem*F gene product showing that the polypeptide has a hydrophylic nature.

CHAPTER 6

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