REACTION CENTRE AND LIGHT HARVESTING GENES OF RHODOBACTER SPHAEROIDES

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ABSTRACT

The photosynthetic bacterium Rhodobacter sphaeroides has been used as a model system for studying the LH1 and LH2 light harvesting (antenna) and reaction centre complexes which make up the photosynthetic apparatus.

The genes encoding the LH2 antenna have been cloned using a synthetic oligonucleotide derived from the published polypeptide sequence. The genes have been sequenced and are part of an operon, transcribed in the order B,A. Transfer of these genes to a mutant lacking LH2 restores the complex to this mutant.

The effect of oxygen on the relative levels and sizes of mRNA encoding the LH2, LH1 and reaction centre L and M polypeptides has been studied. Over the 6 hours of the experiment bacteriochlorophyll levels increased at least 100-fold. No transcripts for the photosyntheteic components were detectable at the start of the induction. After two hours transcripts from the puf operon (LH1 and reaction centre) had reached a maximum, and were 2.7 and 0.5 kb in length. The transcript encoding LH2, estimated to be 0.55 kb, had reached a maximum after 6 hours.

A deletion insertion mutant lacking LH2 has been constructed by using homolgous recombination to replace a 950 bp BamHI fragment encoding pucA and B with a gene for kanamycin resistance. This mutant has been characterised and does not contain the genes or LH2 polypeptides. The LH2 complex was restored to this mutant by transfer of pucA and B.

The 5' end of the puf operon has been sequenced, on a fragment which includes the LH1 genes and the start of the reaction centre L gene. Two open reading frames, denoted puf Q and puf R have been found upstream of the LH1 genes. Northern blot analysis has shown that these genes are in fact transcribed and are part of the puf operon. The 5' end of the puf transcript has been mapped by mung bean nuclease.

A stable mutant lacking LH1 has been constructed by engineering a specific frame shift mutation into the puf B gene. This mutant has been characterised and been shown to lack any detectable amounts of the LH1 α and β apoproteins.

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PRESENTATION OF DATA

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1) Cloning and oxygen regulated expression of genes coding for the reaction centre and light harvesting polypeptides of Rhodopseudomonas sphaeroides. Mark K. Ashby, Shirley A. Coomber and C. Neil Hunter in 'Progress in Photosynthesis Research' (ed. J. Biggins) vol. IV p 733-736 (Martinus Nijhoff 1987).

2) Cloning and oxygen regulated expression of genes for the bacteriochlorophyll biosynthetic pathway in Rhodopseudomonas sphaeroides. Shirley A. Coomber, Mark K. Ashby and C. Neil Hunter in 'Progress in Photosynthesis Research' (ed. J. Biggins) vol. IV p 737-740 (Martinus Nijhoff 1987).

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

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

5) Characterisation and complementation of a mutant of Rhodobacter sphaeroides with a chromosomal deletion in the light harvesting (LH2) genes. J. Grant Burgess, Mark K. Ashby and C. Neil Hunter. In preparation for submission to J. Gen. Microbiol.

6) Chromosomal deletion of the genes encoding the reaction centre and LH1 antenna polypeptides of Rhodobacter sphaeroides. Analysis of the puf operon. Mark K. Ashby, J. Grant Burgess, Shirley A. Coomber and C. Neil Hunter. In preparation for submission to EMBO J.

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1) The oxygen regulated expression of genes coding for reaction centre and light harvesting polypeptides in Rhodopseudomonas sphaeroides M.K. Ashby, S.A. Coomber and C.N. Hunter. AFRC meeting on Photosynthesis Aberystwyth 15-18 April 1986.

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C O N T E N T S

LIST OF FIGURES 10

LIST OF TABLES 12

ABBREVIATIONS 13

1. INTRODUCTION 14

1.1. BACTERIAL PHOTOSYNTHESIS 14

1.2. THE ORGANISATION OF PIGMENT PROTEIN COMPLEXES IN MEMBRANES 15

1.2.1. The Structure of the photosynthetic unit 15
1.2.2. Size of the photosynthetic unit 17
1.2.3. Energy transfer 19

1.3. STRUCTURE OF PIGMENT PROTEIN COMPLEXES 22

1.3.1. Light Harvesting complexes 22
1.3.2. Reaction centre complexes 26

1.4. ORGANISATION OF THE GENES ENCODING THE PHOTOSYNTHETIC APPARATUS 29

1.4.1. Genetic mapping 29
1.4.2. Analysis of cloned genes 31

1.5. REGULATION OF PHOTOSYNTHETIC MEMBRANE ASSEMBLY 34

1.5.1. Assembly of the photosynthetic apparatus 34
1.5.2. Regulation of gene expression 37
1.5.2.1. Pigment biosynthesis genes 37
1.5.2.2. Genes for pigment binding proteins 38

1.6. SUMMARY OF RESEARCH AIMS 45

2. MATERIALS AND METHODS 58

2.1. SOLUTIONS AND MEDIA 58

2.2. BACTERIAL STRAINS 58

2.3. PLASMIDS 58

2.4. MICROBIAL TECHNIQUES 59

2.4.1. Growth of E.coli 59
2.4.2. Growth of Rb.sphaeroides 59
2.4.3. Plasmid transfer 59

2.4.4. Antibiotic concentrations 59
2.4.5. Induction of bacteriochlorophyll biosynthesis 59

2.5. DNA MANIPULATION 60

2.5.1. Preparation of plasmid DNA 60
2.5.2. Preparation of genomic DNA 60
2.5.3. Preparation of competant cells 61
2.5.4. Transformation of competant cells 61
2.5.5. Restriction enzyme digestion 61
2.5.6. Ligation of DNA fragments 62
2.5.7. Blunt Ending of DNA with 5' overhangs 62
2.5.8. Dephosphorylation of DNA 62
2.5.9. ^{32}P-Labelling of DNA 63
2.5.10. Agarose gel electrophoresis 63
2.5.11. Purification of DNA fragments from agarose gels 64
2.5.12. Southern blotting 64
2.5.13. Lysed colony filters 65
2.5.14. Southern and colony hybridisation 65
2.5.15. Preparation of single stranded DNA 65
2.5.16. Sequencing of single stranded DNA 67

2.6. RNA MANIPULATION 68

2.6.1. Preparation of RNA 68
2.6.2. Northern analysis of RNA 68
2.6.3. Quantification of signals on autoradiographs 68
2.6.4. 5' end mapping of RNA 68

2.7. PROTEIN MANIPULATION 69

2.7.1. Spectroscopy 69
2.7.2. Preparation of membranes 69
2.7.3. SDS-PAGE 69

3. CLONING, NUCLEOTIDE SEQUENCE AND TRANSFER OF GENES FOR THE B800-850 LIGHT HARVESTING COMPLEX OF RHODOBACTER SPHAEROIDES 74

3.1. INTRODUCTION 74

3.2. RESULTS 74

3.3. DISCUSSION 77

4. EFFECT OF OXYGEN ON LEVELS OF mRNA CODING FOR REACTION-CENTRE AND LIGHT-HARVESTING POLYPEPTIDES OF RHODOBACTER SPHAEROIDES 83

4.1. INTRODUCTION 83

4.2. RESULTS 84

4.3. DISCUSSION 86

5. CHARACTERISATION AND COMPLEMENTATION OF A MUTANT OF RHODOBACTER SPHAEROIDES WITH A CHROMOSOMAL DELETION OF THE LH2 GENES 92

5.1. INTRODUCTION 92

5.2. RESULTS 93

5.3. DISCUSSION 95

6. ANALYSIS OF THE PUF OPERON 102

6.1. INTRODUCTION 102

6.2. RESULTS 104

6.3. DISCUSSION 107

7. CONSTRUCTION OF A MUTANT OF RB.SPHAEROIDES LACKING THE LH1 ANTENNA COMPLEX 121

7.1. INTRODUCTION 121

7.2. RESULTS 123

7.3. DISCUSSION 126

8. REFERENCES 134

LIST OF FIGURES

1.1. Distribution of photosynthetic bacteria in a 'typical' meromictic lake. 47

1.2. Classification of photosynthetic bacteria. 48

1.3. Schematic representation of light harvesting apparatus of *Rb.sphaeroides* 49

1.4. Models for the arrangement of antenna and reaction centre complexes of *Rb.sphaeroides*. 50

1.5. Comparison of primary structures of the antenna polypeptides from purple photosynthetic bacteria. 51

1.6. Schematic representation of LH1 and LH2 from *Rb.sphaeroides* 52

1.7. Schematic representation of the reaction centre polypeptides and cofactors. 53

1.8. Genetic map of the photosynthetic gene cluster of *Rb.sphaeroides*. 54

1.9. Restriction maps of *Rb.sphaeroides puf*, *puc* and *puh* operons. 55

3.1. Southern blot analysis of recombinant clones. 79

3.2. Restriction map of pMA81. 80

3.3. Nucleotide sequence of the 1,078bp *Xho*I-*Pvu*II fragment. 81

3.4. Absorbance spectra of M21, M2181 and wild type *Rb.sphaeroides*. 82

4.1. Absorbance spectra of *Rb.sphaeroides* undergoing pigment induction. 88

4.2. Restriction maps of pJWI and pMA81. 89

4.3. Northern blots of *Rb.sphaeroides* RNA following pigment induction. 90

4.4. Graph showing changes in levels of bacteriochlorophyll and mRNA for photosynthetic complexes. 91

5.1. Construction of DBCI. 98

5.2. Southern hybridisation of DBCI genomic DNA. 99

5.3. Absorbance spectra of diluted membrane preparation from DBCI, DBCI.81 and 8253. 100

5.4. SDS-PAGE of solubilised membranes from 8253, DBCI and DBCI.81. 101

6.1. Restriction map of pGBI and the chromosome of DPFI. 111

6.2. Absorbance spectra of membranes purified from DPFI, DPFI(pSRC6), 8253, MDPFI, MDPFI(pSRC6) and M21. 112

6.3. SDS-PAGE of membranes purified from 8253, DPFI, DPFI (SRC6), M21, MDPFI and MDPFI(pSRC6). 113

6.4. Restriction map of the 2,228bp BamHI-KpnI fragment, showing fragments used for sequencing and Northern blot analysis. 114

6.5. Nucleotide sequence of the 2,228bp BamHI-KpnI fragment. 115

6.6. Northern blot analysis of Rb.sphaeroides RNA probed with puf operon specific DNA fragments. 116

6.7. Mung bean nuclease analysis of Rb.sphaeroides RNA. 117

6.8. Restriction map of the 2,228bp BamHI-KpnI fragment showing the pufQ and pufR genes and mRNA species mapped. 118

6.9. Homology between Rb.sphaeroides and Rb.capsulatus puf operon promoters. 119

6.10. Homology between the deduced amino acid sequences of pufR and pufQ from Rb.sphaeroides and Rb.capsulatus. 120

7.1. Construction of a mutant of Rb.sphaeroides deficient in LH1. 129

7.2. Absorbance spectra of membranes purified from: DPFI, MA3 and 8253. 130

7.3. Absorbance spectra of membranes purified from: MA3 and NF57. 131

7.4. SDS-PAGE of membranes purified from: 8253, DPFI and MA3. 132

7.5. Northern blot analysis of RNA prepared from 8253 and MA3 and probed with a puf operon specific DNA fragment. 133

LIST OF TABLES

1.1. Domain sizes of LH1 and LH2 complexes of *Rb.sphaeroides*. 56

1.2. Kinetics of energy transfer within the photosynthetic apparatus of *Rb.sphaeroides*. 57

2.1. Constituents of M22+ media. 70

2.2. Bacterial strains. 71

2.3. Plasmids. 72

2.4. Antibiotic concentrations. 73

ABBREVIATIONS

bch	bacteriochlorophyll biosynthesis gene
bchl	bacteriochlorophyll
bchl 800,850,875	antenna bacteriochlorophyll absorbing at 800,850 and 875nm respectively
bp	base pair(s)
CM	cytoplasmic membrane
crt	carotenoid
crt	carotenoid biosynthesis gene
dATP	2'- deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGPT	2'-deoxy-guanosine-5'-triphosphate
DNA	deoxyribonucleic acid
dNTP's	mixture of dATP, dCTP, dGTP, dTTP.
dTTP	2'-deoxy-thymidine-5'-triphosphate
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
ICM	intracytoplasmic membrane
IPTG	isopropylthiogalactopyranoside
kb	kilobase pair(s)
LH1	light harvesting complex 1
LH2	light harvesting complex 2
mRNA	messenger RNA
Rb.sphaeroides	Rhodobacter sphaeroides
Rb.capsulatus	Rhodobacter capasulatus
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate polyocrylamide gel electrophoresis
X-gal	5-bromo-4-chloro-3indoyl- -D-galactopyranoside

1. INTRODUCTION

1.1. BACTERIAL PHOTOSYNTHESIS

The energy for the production of virtually all biological matter ultimately comes from photosynthesis, which is a process whereby solar energy is used by plants and photosynthetic bacteria to reduce carbon dioxide to more complex organic molecules, with water (as is the case with plants and cyanobacteria) or other molecules such as hydrogen sulphide as the hydrogen donor. This reaction can be simply represented in the general formula shown below.

$$CO_2 + 2\,H_2A \xrightarrow{\text{Light}} (CH_2O) + 2A + H_2O$$

CO_2	$2\,H_2A$	(CH_2O)	$2A$
Hydrogen acceptor	Hydrogen donor	Reduced acceptor	Dehydro-genated donor

Photosynthetic bacteria are represented by a diverse range of micro-organisms. The cyanobacteria, like plants, carry out oxygenic photosynthesis, have two photosystems and use water as the electron donor, evolving oxygen as the final oxidation product. Phototrophic green and purple bacteria are much simpler, having only one photosystem and they carry out anoxygenic photosynthesis. The phototrophic green and purple bacteria are grouped together in the order Rhodospirillales and are represented by the sub-orders Chlorobineae and Rhodospirillineae which are depicted in figure 2.

Cyanobacteria and green and purple bacteria are often found together, occupying distinct ecological niches, due to their particular characteristics. In a typical meromictic lake, in which only the top layer of water will be turning over, distinct bands can be observed for growth of photosynthetic bacteria (Pfennig 1978), as illustrated in figure 1. In the top layer one would find cyanobacteria, which require oxygenic conditions and plenty of light, absorbing wavelengths less than 690nm. Below this fairly oxygenated layer the water becomes low in oxygen. At the bottom of the lake there is an anaerobic layer. At the bottom

of this anaerobic layer near the decaying mud the green photosynthetic bacteria are found. They are largely non-motile, tolerant to low temperatures and low light, and require high sulphide concentrations, while absorbing light in the range 700 - 760 nm. The purple photosynthetic bacteria are found in between the anaerobic and aerobic layers and being mostly motile can move to their ideal environment, which is intermediate light intensity and low sulphide concentrations, while absorbing light above 800nm and being able to tolerate higher oxygen tensions than green bacteria.

Purple bacteria can be divided into two distinct groups. The purple sulphur bacteria (Chromatiaceae) and the purple non sulphur bacteria (Rhodospirillaceae). Purple sulphur bacteria are strictly anaerobic requiring sulphide as the electron donor. Purple non-sulphur bacteria are by far the most diverse group, which in addition to being able to carry out anaerobic photosynthesis, have varying abilities to respire aerobically. A large range of compounds can be used by members of this family for reducing power, including hydrogen sulphide, hydrogen and a large number of simple organic molecules like acetate and pyruvate (Truper and Pfennig, 1978). The genus and species of the family Rhodospirillaceae are shown in figure 2. The study of Rhodospirillaceae has only been possible due to the pioneering work of van Niel (1944) and Pfennig (1967) in working out the culture techniques to enrich and isolate these photosynthetic bacteria from the inocula, and to characterise and classify the strains isolated.

1.2. THE ORGANISATION OF PIGMENT PROTEIN COMPLEXES IN MEMBRANES

1.2.1. The Structure of the Photosynthetic Membrane

The photosynthetic apparatus of all photosynthetic organisms either exclusively, or in part consists of hydrophobic membrane bound pigment binding proteins (Zuber, 1986; Barber, 1987) and is made up of reaction centres where change separation occurs, and an antenna system which is a network of specialised pigment binding protein complexes which absorb photons of light and efficiently transfer this

energy as excitations to the reaction centre. The photosynthetic apparatus of green algae and higher plants consists of a large number of transmembrane pigment binding protein complexes situated in specialised organelles called thylakoids (Zuber, 1985), using chlorophyll as the major pigment. Cyanobacteria and red algae also house their photosynthetic apparatus in the thylakoid membrane, but have an additional antenna system arranged on the surface of the thylakoid membrane, called a phycobilisome. The photosynthetic apparatus of photosynthetic purple bacteria is located in specialised invaginations of the cytoplasmic membrane (CM) called the intracytoplasmic membrane system (ICM) (Niederman and Gibson, 1978), being made up of the reaction centre and only two antenna pigment protein complexes, the major pigments being bchl and carotenoids. Green photosynthetic bacteria, in addition to possessing ICM bound reaction centres and antenna, have extra antenna complexes both on the surface of the membrane, and located in a structure called the chlorosome bound to the membrane (Zuber, 1985).

The photosynthetic apparatus of purple photosynthetic bacteria, is relatively simple when compared to other photosynthetic organisms, and has therefore been well studied. Photosynthetic membrane assembly can be induced in the CM of Rb.sphaeroides by a reduction in the oxygen tension of aerobic culture media (Niederman et al., 1976). At high oxygen tensions small invaginations of the CM are present, containing small amounts of functional pigment protein, which are thought to be sites of initiation of growth of the CM invaginations (Niederman et al., 1979; Hunter et al., 1982) yet to become the mature ICM (see section 5, this chapter for a discussion of the regulation of photosynthetic membrane assembly). The photosynthetic apparatus in the ICM contains three intramembrane pigment protein complexes, the reaction centre (Clayton and Wang, 1971; Feher, 1971) and two light harvesting antenna complexes LH1 and LH2 (Cogdell and Thornber, 1980), with

reaction centres surrounded by LH1 complexes, which are in turn surrounded by a larger number of LH2 complexes (Vos et al., 1986), as depicted in figure 3. Light energy is absorbed by LH2 and LH1 antennae, and excitation energy is then passed to the reaction centre, where change separation takes place (van Grondelle, 1985).

The formation and shape of the ICM vesicles is linked to the insertion of the pigment protein complexes into the CM, and probably more specifically to the insertion of the LH2 complexes into the CM (Hunter et al., 1988b). Electron microscopy of mutants lacking LH2 antenna has shown a tubular ICM system, rather than vesicles (Hunter et al., 1988b, Kiley et al., 1988), suggesting that LH2 plays a direct role in changing the morphology of intracellular membranes from a tubular to a spherical structure. Mutants lacking LH1 antenna have also been studied. Rb.sphaeroides mutants lacking LH1 are photosynthetic (Meinhardt et al., 1985; Jackson et al., 1987), whereas such mutants of Rb.capsulatus are not photosynthetic (Jackson et al., 1986; Jackson et al., 1987), indicating that LH1 is necessary for the correct insertion of reaction centres into the ICM, at least in Rb.capsulatus.

1.2.2. Size of the Photosynthetic Unit

The assembly of a number of reaction centres surrounded by the LH1 and LH2 antenna complexes forms a distinct functional domain or photosynthetic unit (Vos et al., 1988). The size of this unit can be estimated by a technique called singlet-singlet annihilation, where the probability that two excitations will collide, annihilating one depends on the size of the domain in which they occur (van Grondelle et al., 1983; Vos et al., 1987). Both isolated LH1 and LH2 complexes (van Grondelle et al., 1983) and domains in isolated ICM's have been studied. (Hunter et al., 1985).

Detergent purified LH1 and LH2 complexes have been found to aggregate into fairly well defined sizes (van Grondelle et

al., 1983). LH1 forms domains of about 8 bchl and LH2 either > 25 bchl or > 600 bchl depending on the detergent used. This, and other data suggested a possible arrangement for the antenna of a 'lake' of LH2 made up of units containing 25 to 30 bchl's surrounding LH1, which in turn could contain about 100 - 140 bchl molecules, a total of 12 - 18 LH1 complexes could then surround a small number of reaction centres. (Monger and Parson, 1977; Vos et al., 1986; Vos et al., 1988).

Studies of intact ICM preparations (Hunter et al., 1985; Vos et al., 1986), have shown that in the mature photosynthetic ICM the light harvesting network in each invagination contains about 3,000 bchl molecules, which connect about 30 reaction centres; each reaction centre is separated on average by 15nm so therefore a photosynthetic unit effectively encompasses an ICM invagination. Annihilation studies at 4K (Vos et al., 1986) enable the domains observed at room temperature to be resolved into smaller units. Rhodospirillum rubrum contains only one light harvesting complex which at room temperature forms a network containing about 1000 bchl molecules, however at 4K this size decreases 8 fold to 150 bchl molecules, suggesting that the light harvesting molecules are ordered into an interconnecting structure containing about 150 bchl molecules. In Rb.sphaeroides at 4K LH1 domains were estimated to contain about 100 bchl 875 molecules, and LH2 domains, about 45 bchl 850 molecules, compared to about 300 bchl 850 molecules at room temperature (Vos et al., 1988). This led to the proposal of a model whereby about 5 LH2 domains (each containing about 45 bchl 850 molecules) surround an LH1 domain (containing about 100 bchl 875 molecules) which surround and interconnect about 4 reaction centres. A number of LH1- reaction centre 'cores' are interconnected by LH2.

This model has been further refined by studies on mutants lacking one or the other antenna complex (Vos et al., 1988;

Hunter and van Grondelle, 1988). M21 which contains reaction centre and LH1, M2192 which contains LH1 and NF57 which contains only LH2 have been studied at room temperature and at 4K (see table 1). The smallest domain for LH1 at 4K was nearly identical for wild type, M21 and M2192 (about 100 bchl 875 molecules) but at room temperature M2192 was unable to form significantly larger domains, suggesting that reaction centres are necessary for the correct formation of LH1 domains. LH1 domains in M21 at room temperature were not as large as in wild type, possibly due to the restrictions imposed by the lipid-rich tubular ICM structure (Hunter et al., 1988b). NF57, which contains only LH2 antenna could form large domains at room temperature, greater than 360 bchl 850 molecules, which at 4K could be resolved to domains of about 30 bchl 850 molecules.

In the refined model (see figure 4) 4 reaction centres are surrounded by about 100 bchl 875 molecules (6bchl per minimal unit); these cores would then be surrounded and interconnected by 5 or 6 clusters of LH2 complexes containing about 30 bchl 850 and 15 bchl 800 molecules per cluster (9 bchl per minimal unit). These domains would link up to form a light harvesting network in a single ICM invagination, containing a total of 3,000 bchl molecules and about 30 reaction centres.

1.2.3. Energy Transfer

The transfer of excitation energy within and between light harvesting complexes has been studied in order to elucidate the mechanisms of efficient light harvesting and energy transfer in bacterial antennae. The optical properties of pigment protein complexes in the ICM have been studied by a variety of techniques, including circular and linear dichroism, excitation spectra of fluorescence polarisation and time resolved picosecond absorption recovery. They reveal the details of energy transfer between identical pigments and between non identical pigments, usually down an energy gradient (van Grondelle and Amesz, 1986; Sundstrom et

al., 1986) identifying a fast initial transfer betwen similar bchl 800 molecules. Excitation of bchl 800 is very short lived and the energy is rapidly transferred to bchl 850 (about 1ps) within LH2. Transfer between pigment pools from LH2 to LH1 is slower (about 30ps) and then transfer from LH1 to reaction centre is slow again (about 70ps). Their results suggested that transfer of excitation energy was fairly ordered, passing through pigments of successively higher absorbance, before trapping by the reaction centre (Sundstrom et al., 1986). LH2 and LH1 complexes were also found not to be spectrally homogenous. Earlier studies of LH1 complexes in the membrane and of isolated complexes showed the presence of an extra pigment absorbing at 896 nm, comprising about 15% of the bchl absorption of LH1 (Bolt et al., 1981; Kramer et al., 1984a). Efficient and fast energy transfer was demonstrated from bchl 875 to bchl 896, which at low temperatures was irreversible. Further analysis has suggested that bchl 896 molecules are clustered around, and in contact with the reaction centres (see figure 4), and function to transfer excitation energy from bchl 875 to the reaction centre (Vos et al., 1986; Hunter and van Grondelle, 1988; Bergstrom et al., 1988). By selectively exciting bchl 896 at 4K, the emissions were found to be strongly polarised, indicating a certain degree of orientation of the bchl 896 molecules around the reaction centre, which are connected to each other only by bchl 875 (van Grondelle et al., 1987). It was proposed that bchl 896 is needed as a focus for the antenna to transfer energy to the reaction centre.

The availability of Rb.sphaeroides mutants, deficient in one or other antenna complex has enabled more detailed analysis of energy transfer within each specific antenna in the ICM, and in particular the characterisation of minor bchl species in LH1 and LH2. Mutant strain M21 which lacks LH2 and M2192 which lacks LH2 and reaction centres were shown to contain a minor bchl component in LH1 absorbing at 899nm, presumably equivalent to bchl 896 in wild type Rb sphaeroides

membranes (van Dorssen et al., 1988; Vos et al., 1988). Spectral analysis of LH2 complexes in mutant NF57, which lacks LH1 and reaction centres has shown a minor absorbing bchl at 870nm at 4K, comprising about 15% of the total bchl content of LH2, which is totally absent in detergent purified LH2 complexes (van Dorssen et al., 1988; Hunter et al., 1988c). At room temperature most of the emission from LH2 comes from bchl 850, but when cooled to 4K, the bchl 870 emission becomes predominant. Excitation spectra at 4K indicated that most energy is efficiently transferred from bchl 800 to bchl 850 and then to bchl 870, which can then 'funnel' the excitation to bchl 875 of LH1.

The important features of the kinetics of energy transfer within the photosynthetic membrane are shown in table 2.

1.3. STRUCTURE OF THE PIGMENT PROTEIN COMPLEXES

1.3.1. Light Harvesting Complexes

The light harvesting antenna of photosynthetic bacteria contains the majority of the pigment molecules in the photosynthetic apparatus of the cell, specifically bound in pigment protein complexes. The antenna complexes of purple non-sulphur photosynthetic bacteria are exclusively membrane bound, and the bacteria can be divided into three groups depending on their characteristic absorbance spectra Group 1 : Rhodospirillum rubrum, Rhodopseudomonas viridis (880nm, 1015nmrespectively); group 2: Rb.sphaeroides, Rb.capsulatus, Rb.gelatinosa (800nm, 850nm, 875nm); group 3: Chromatium vinosum, Rb.acidophila (800nm, 820nm, 850nm, 875nm) (Zuber, 1986b). There are two types of light harvesting complex, LH1 and LH2 (Cogdell 1986). The LH1 complex, for example the B880 complex of Rhodospirillum rubrum (representing the sole light harvesting complex) and the B875 of Rb.sphaeroides, is usually strongly associated with the reaction centre, and characterised by a single bchl absorption maximum. The LH2 complex, present in most photosynthetic bacteria, for example B800-850 of Rb.sphaeroides, can be present in variable amounts in comparison to the reaction centre and is characterised by having two bchl absorption maxima. The variation in level of the LH2 complex is associated with adaption of the photosynthetic apparatus to light intensity and oxygen tension (see section 5, this chapter).

In order to isolate intact light harvesting complexes it is necessary to treat chromatophore preparations with detergent to disrupt the phospholipid membrane, and solubilise the hydrophobic protein complexes (Cogdell and Thornber, 1980), which can then be separated by a number of different fractionation procedures. The detergent used for the isolation is important as it has to be strong enough to disrupt the membrane, but still leave the polypeptides intact. The isolation of complexes has shown that the absorption at 800nm, 850nm and 875nm in Rb.sphaeroides is

due to two different complexes, LH1 - 875nm and LH2 - 800nm and 850nm (Clayton and Clayton, 1972; Broglie et al., 1980). The bchl 800 moiety of LH2 was found to be particularly sensitive to the detergent used (Clayton and Clayton, 1981).

Analysis of the protein and pigment content of these isolated complexes shows that they are made up of equimolar amounts of two low molecular weight hydrophobic apoproteins (50 to 60 amino acids) with bchl and usually carotenoid as the bound pigment (Zuber, 1985). The primary structures of a number of light harvesting apoproteins have been ascertained, and found to contain a number of common features (Zuber, 1986b). Three distinct domains are present, a central hydrophobic core of 20 to 30 amino acids, which could form a membrane spanning α helix, with polar N and C terminal domains (see figure 5). The hydrophobic domain contains one or two conserved histidine residues which were postulated to be responsible for binding bchl (Zuber, 1985). Recently it has been shown that asparagine may be responsible for binding bchl in the closely related bacterium Ectothiorhodospira halochloris (Wagner-Huber et al., 1988).

Estimates of the pigment content of LH1 and LH2 complexes put the ratios of pigments in LH2 at 3 bchl : 1 carotenoid (Clayton and Clayton, 1972; Cogdell and Crofts, 1978) but was subsequently shown to be 2 : 1 (Broglie et al., 1980). For LH1 this ratio is 2 bchl : 2 carotenoids per $\alpha\beta$ pair of polypeptides (Broglie et al., 1980). The absorbance maximum of bchl when extracted with organic solvent is about 770nm, which is 30nm to 120nm shorter in wavelength than that of bchl in intact complexes. Such large red shifts in the absorbance of bchl are due to the environment created by the apoproteins to which the pigment is non-covalently bound (Zuber, 1985). LH2 has two different bchl absorbance maxima at 800nm and 850nm, which have been attributed to different bchl molecules, in particular in view of the fact that bchl

800 can selectively and reversibly be removed, leaving bchl 850 (Clayton and Clayton, 1981), indicating that these are separate bchl molecules, which have different red shifts due to the micro-environment created by the polypeptides. In addition, a carotenoidless mutant of Rb.sphaeroides, R26.1, has an amino acid substitution in the LH2 α polypeptide causing it to lose bchl 800, while still retaining bchl 850 (Davidson and Cogdell, 1981; Theiler et al., 1984).

A good picture of the structure of light harvesting complexes in the membrane can be ascertained from studying the spectral properties of isolated complexes, and complexes in the membrane as well as knowledge of the stoichiometry of the components, and theoretical considerations derived from the primary structure of the apoproteins. Low temperature fluorescence polarisation spectra of isolated LH2 complexes (Kramer et al., 1984b) indicated the orientation of the pigments in the complex. The bchl 800 molecules are orientated parallel to each other near the surface of the membrane, whereas the bchl 850 molecules appeared to be perpendicular to the membrane. The carotenoids also seem to fall into two groups with different absorption maxima; a carotenoid orientated parallel to the membrane transferring energy to bchl 800, and a carotenoid lying nearly perpendicular to the membrane transferring energy to bchl 850. Polarization of the flourescence from isolated LH2 complexes indicated that the smallest complex obtainable contained 8 to 10 bchl 850 molecules, (Kramer et al., 1984b) which together with knowledge of the primary structure of the polypeptides suggested a minimal unit for the complex consisting of 2 α and 2 β polypeptides, each spanning the membrane, together with 4 bchl 850, 2 bchl 800 and 3 carotenoid molecules. Low temperature spectra of isolated LH1 complexes (Kramer et al., 1984a) shows that all of the bchl 875 molecules are oriented the same, perpendicular to the membrane, even though a small percentage of the bchl was found to absorb at a longer wavelength. A minimal unit for

LH1 appeared to contain 3 α and 3β polypeptides with 6 bchl 875 molecules and carotenoids (Kramer et al., 1984a).

The model of the LH2 complex has been modified to contain 3 α and 3β polypeptides, 6 bchl 850, 3 bchl 800 and 6 carotenoid molecules (Hunter et al., 1988a) in view of the homology between the primary structure of LH1 and LH2 apoproteins (Zuber, 1987), with the bchl 850 molecules slightly tilted with respect to the perpendicular to the membrane, and the bchl 800 molecules slightly tilted with respect to the cytoplasmic face of the membrane (see figure 6a). The orientation of the bchl 875 molecules in LH1 has also been tilted with respect to the perpendicular to the cytoplasmic surface of the membrane (see figure 6b). The structures presented in these models await X-ray crystallographic studies. Analysis of mutants with only LH2 or LH1 and reaction centres as the pigment protein complexes has indicated that both LH1 and LH2 contain minor longer wavelength bchl components, bchl 896 in LH1 (Vos et al., 1988; van Dorssen et al., 1988) which may be bchl molecules in a special environment with the bchl 870 being involved in energy transfer from LH2 to LH1 and bchl 896 transferring energy from LH1 to the reaction centre.

An indication of the influence of the polypeptides and the external aqueous environment has recently been demonstrated in the isolated B873 light harvesting complex of Rhodospirillum rubrum (Parkes-Loach et al., 1988). The absorption of the isolated complex could be shifted from 873nm down to 829nm by detergent treatment, and then the bchl removed by further treatment to leave a stable complex, and free bchl absorbing at 777nm. The 820nm and 873nm complexes could be reversibly reformed from the constituent polypeptides and bchl, indicating the influence of the polypeptides on bchl, although the effects of the detergent on free bchl cannot be ruled out.

The necessity of having bchl in order to form a stable complex has been demonstrated in Rb.capsulatus mutants unable to synthesise bchl in which stable light harvesting complexes do not form, and the polypeptides are rapidly degraded as they are synthesised (Klug et al., 1986).

1.3.2. Reaction Centre Complexes

The reaction centres of all photosynthetic organisms lie at the centre of the photosynthetic unit within a lipid membrane (see figure 3) taking excitation energy from the light harvesting apparatus to a 'special pair' of bchl molecules, which are able to eject an electron, which is transferred through a series of electron carriers, converting the redox energy into chemical energy (Feher and Okamura,1978). For the structure of the reaction centre, one does not have to rely on models as the complex has been successfully crystallised from Rhodopseudomonas viridis and Rb.sphaeroides, and the crystals used to obtain the 3-dimensional structure of each complex, at a resolution of2.9Å and 2.8 Å respectively(Michel et al.,1986;Allen et al.,1987a).

The first reaction centre was obtained from a carotenoidless strain of Rb.sphaeroides by detergent treatment of chromatophores (Clayton and Wang, 1971; Feher, 1971), and characterised by absorption at 800nm and 860nm. This complex was found to contain equimolar amounts of 3 polypeptides, heavy (H), medium (M) and Light (L), based on their electrophoretic mobility (Clayton and Haselkorn, 1972; Steiner et al., 1974). A photochemically active core containing the L and M polypeptides was obtained, indicating that this is the unit which binds the pigments, bacteriochlorophyll and bacteriopheophytin (bphe), as well as quinone (Q) molecules, and iron (Steiner et al., 1974).

The X-ray crystal structure analysis of reaction centres from R.viridis and Rb.sphaeroides (Deisenhofer et al., 1985; Allen et al., 1987a) shows that both the L and M subunits contain 5 transmembrane α helices. The structure

(and the primary sequence) of the L and M apoproteins is remarkably similar, being related by a 2 fold rotational symmetry axis, whereas the H subunit has only one transmembrane α helix, and forms a 'cap' structure overlying the L and M polypeptides (see figure 7a). In addition, the reaction centre isolated from R.viridis contains a c-type cytochrome which sits on the periplasmic surface of the membrane on top of the reaction centre. The structure of the cofactors in the reaction centres from Rb. sphaeroides and R.viridis is similar (Michel et al., 1986; Allen et al., 1987b), and these are arranged along two branches of the L and M subunits called the A and B branch, related by a two-fold symmetry axis, the cofactors being found at a point where a helical region of the L and M subunits meet, which in Rb.sphaeroides and R.viridis are composed of 4 bchl, 2 bphe, 2 quinones and one haem iron molecule (see figure 7b). Excitation energy is transferred from the light harvesting apparatus to the special pair of bchl molecules of the A and B branch near the periplasmic surface of the reaction centre; the special pair quickly eject an electron which travels down the A branch to bphe A (about 4 picoseconds), which may or may not be influenced by the bchlA monomer. The electron is then passed further along the A branch to QA (about 200 picoseconds) and then fairly slowly onto QB on the B branch (about 200 nanoseconds). The electron would then pass onto a b-andc-type cytochromes in passing along the electron transport chain. The electron 'hole', left in the reaction centre is filled by electron transfer from a c-type cytochrome. The role of the iron atom is unclear, but it may play a structural role in binding the L and M subunits together.

The orientation of the reaction centre in the membrane cannot be determined by X-ray diffraction as the water and detergent in the crystals do not form a diffraction pattern, but predictions can be made by minimising hydrophobic energy within the membrane, and taking into account the dimensions of the complex, and of the phospholipid membrane (Yeates et

al., 1987). The most stable model puts the two-fold axis of the complex perpendicular to the surface of the membrane, with the average tilt for the transmembrane α helices at 22 deg.

Now that the position of most of the amino acids is known in the reaction centre, experiments using the technique called site directed mutagenesis can be used to probe the role and importance of each amino acid in the polypeptide, by changing the amino acids encoded in the genes. This technique has recently been used to change isoleucine 229 of the reaction centre L subunit of Rb.capsulatus to seventeen other amino acids, engineering herbicide resistance in six of the mutations (Bylina and Youvan, 1987).

1.4. ORGANISATION OF THE GENES FOR THE PHOTOSYNTHETIC APPARATUS

1.4.1. GENETIC MAPPING

The genes for the photosynthetic apparatus in some Rhodospirillaceae are amenable to genetic analysis because, in addition to exhibiting photoheterotrophic growth, these bacteria can grow by aerobic respiration (Pfennig, 1978), thus enabling non-photosynthetic mutants to be isolated and propagated in aerobic culture. Genetic analysis has centred on Rb.capsulatus and Rb.sphaeroides, and so the work discussed will be that done on these two organisms.

The gene transfer agent of Rb.capsulatus, a generalised transducing phage which randomly packages chromosomal DNA, was used to transfer small fragments of DNA (about 4.5kb) to specific mutants with lesions in pigment biosynthesis genes. Complementation of some of these mutants allowed a small cluster of genes to be linked together (Yen and Marrs, 1976; Scolnik et al., 1980) in the order crt A, crt B, crt C, crt G, crt D, crt E, crt F, bch C, bch A. This technique has the drawback in that it is random, and only small fragments of DNA are packaged, therefore only closely linked genes can be mapped. This technique is also specific for Rb.capsulatus, and no comparable transfer mechanisms have been found for other photosynthetic bacteria.

Larger fragments of DNA have been mobilised into mutants by using promiscuous R plasmids both in Rb.sphaeroides (Miller and Kaplan, 1978) and Rb. capsulatus (Marrs, 1981). In Rb.capsulatus the linkage map from the gene transfer agent work was confirmed, and in addition a reaction centre gene was shown to be closely linked to this gene cluster (Marrs, 1981). In similar work a derivative of RP1 was used to produce a linkage map of amino acid biosynthetic genes on the Rb.sphaeroides chromosome (Bowen and Pemberton, 1985); the crt/bch gene cluster was mapped between the nitrogen fixation and adenine biosynthesis genes.

More detailed analysis of this photosynthetic gene cluster has been possible using cloned DNA. A 46kb fragment of Rb.-capsulatus DNA (Taylor et al., 1983) cloned on an R-prime plasmid was restriction mapped and a large number of restriction fragments subcloned. These subclones were mobilised into non-photosynthetic mutants of Rb.capsulatus confirming the genetic linkage map of the photosynthetic gene cluster and aligning the linkage map with specific distances in kilobases of DNA. In addition a further 4 bch and 2 rxc (now puf) genes were discovered. The direction of transcription of some of these genes was indicated as some genes were expressed from plasmid borne promoters, but only in one orientation. The carotenoid gene cluster from Rb. sphaeroides (crt A to F) was cloned and mapped (Pemberton and Harding, 1986) within a region of 15kb, organised in a similar order to Rb.capsulatus

Probably the most sensitive technique for mapping clusters of genes is by transposon mapping (Youvan et al., 1982; Zsebo and Hearst, 1984; Hunter, 1988b) whereby mutants are made rather than complemented. This is done by transferring cloned DNA, which contains mapped transposon insertions, into wild type cells to generate mutants, which can then be characterised. This technique has been used to refine the position of genes within the map of the photosynthetic gene cluster of Rb.capsulatus (Zsebo and Hearst, 1984), adding a further possible 2 crt genes, 3 bch genes and one regulatory gene affecting reaction centre gene expression to the map. A transposon map of the photosynthetic gene cluster of Rb.sphaeroides has recently been constructed (Coomber, S.A. personal communication) over a distance of about 45 kb which shows remarkable similarity to the map of this gene cluster from Rb.capsulatus (see figure 8). These results may seem surprising in view of the lack of homology found between the photosynthetic gene clusters from Rb.capsulatus and Rb.sphaeroides (Beatty and Cohen, 1983) when heterologous probing was attempted. The results of Beatty and Cohen must be considered suspect considering the

large amount of homology between the light harvesting and reaction centre polypeptide sequences (Youvan et al., 1984; Brunisholz and Zuber, 1988; Michel and Deisenhofer, 1988). Heterologous probing has been attempted in this laboratory (Coomber, S.A. personal communication) with DNA from the Rb.sphaeroides gene cluster; strong homology was found not only with DNA from Rb.capsulatus but signals were also detected with DNA from other photosynthetic bacteria.

1.4.2. ANALYSIS OF CLONED GENES

Genes for pigment biosynthesis:

The structure of the pigment biosynthesis genes has not been studied in any great detail. The genes for crt and bch synthesis have been mapped into probable operons by transposon mapping of the Rb.capsulatus gene cluster (Zsebo and Hearst, 1984), which also indicated the direction of transcription of these genes. There is no published data available on the sequence of any pigment biosynthetic gene.

Genes for pigment binding proteins:

The genes that encode the reaction centre L and M subunits and the LH1 α and β apoproteins are located on the puf operon which has been mapped close to the bch A gene of Rb.capsulatus and Rb.sphaeroides (Zsebo and Hearst, 1984; Hunter, 1988b; Coomber, S.A. personal communication). The puf operon of Rb.capsulatus (Marrs, 1981) and Rb.sphaeroides (Williams et al., 1983) and Rhodospirillum rubrum (Bélanger et al., 1988) has been cloned and the genes sequenced (Williams et al., 1983; Williams et al., 1984; Youvan et al., 1984; Kiley et al., 1987; Bauer et al., 1988; Bélanger et al., 1988). The order of the genes in the puf operon is puf B, A, L, M and then another open reading frame of unknown function denoted puf X (Youvan et al., 1984; Kiley and Kaplan, 1988); together they span a total of about 2.5kb (see figure 9a). The puf B and A genes of Rb. sphaeroides are 150 and 177 nucleotides long respectively, separated by 13 nucleotides; the puf L and M genes start a further 113 nucleotides downstream, and are encoded by genes of 849 and

927 nucleotides respectively which overlap by 8 nucleotides (Williams et al., 1983; Williams et al., 1984; Kiley et al., 1987). The genes are encoded by two transcripts of about 0.5kb and 2.6kb (Belasco et al., 1985; Zhu and Kaplan, 1985) which encode puf B A and puf B,A,L,M,X respectively, and will be discussed in further detail in section 5 of this chapter.

Recently another puf encoded gene has been found in Rb. capsulatus 156 nucleotides upstream of puf B called puf Q (Bauer et al., 1988); results indicate that this gene is part of the puf operon. The gene is 225 nucleotides long and may be involved in bacteriochlorophyll biosynthesis. This gene and a further gene upstream called puf R has been reported in Rb.sphaeroides (Kiley and Kaplan, 1988), puf R has been suggested to be involved in insertion of LH1 complexes into the ICM (Kiley and Kaplan,1988).

The H subunit of the reaction centre is encoded by the puh A gene on a separate operon called puh (see figure 9b). This operon has been cloned in both Rb.capsulatus (Marrs, 1981) and Rb.sphaeroides (Donohue et al., 1986a; Coomber and Hunter, 1988), and has been sequenced in Rb.capsulatus (Youvan et al., 1984). puh A maps close to the bch L gene, about 40kb from the puf operon (Coomber, S.A. personal communication). The Rb.sphaeroides gene is 780 nucleotides long, and is encoded by two transcripts of about 1.1kb and 1.4kb (Donohue et al., 1986a) which could encode further smaller genes, although more sequence analysis will be required to elucidate this. The Rb.sphaeroides mutant NF57 which lacks LH1 and reaction centres was restored to photosynthetic growth by a clone containing this gene (Hunter and van Grondelle, 1988). This would indicate that the puh A gene or another gene on the puh operon plays a crucial role in the assembly or insertion of the reaction centre-LH1 complex into the ICM.

The genes encoding the LH2 α and β apoproteins, denoted puc A and B, have been cloned and sequenced in both Rb. sphaeroides (Ashby et al., 1987; Kiley and Kaplan, 1987) and Rb.capsulatus (Youvan and Ismail, 1985). The A and B genes are located on the puc operon, and transcribed in the order B, A (see figure 9c); the Rb.sphaeroides B and A genes are 150 and 164 nucleotides long respectively and are separated by a spacer region of 15 nucleotides. The genes from Rb.sphaeroides are encoded by a single transcript of between 0.55kb (Hunter et al., 1987) and 0.64kb (Kiley and Kaplan, 1987), which does not appear to encode any other genes. The puc operon is not present on the 45kb photosynthetic gene cluster (Youvan and Ismail, 1985; Coomber, S.A. personal communication). There is a preliminary report indicating that the puc operon is linked to within 100kb of this cluster on the R' plasmid pWS2 (Kiley and Kaplan, 1988).

Genes for cytochrome apoproteins:

The membrane bound cytochrome b/c_1 complex and extrinsic cytochrome C_2 are involved in cyclic photosynthetic electron flow. The genes for the cytochrome b/c_1 complex have been cloned and sequenced from Rb.capsulatus (Gabellini et al., 1985; Gabellini and Sebald, 1986; Daldal et al., 1987; Davidson and Daldal, 1987a). Although Gabellini's group published their results nominally on Rb.sphaeroides, it has since been realised that they were working on Rb.capsulatus (Davidson and Daldal, 1987b). The genes for the cytochrome b/c_1 complex are located on the pet operon in the order pet A, pet B, and pet C. The gene for the cytochrome C_2 apoprotein is encoded on a separate operon by the cyc A gene, which has been cloned and sequenced from both Rb.capsulatus (Daldal et al., 1986) and Rb.sphaeroides (Donohue et al., 1986b). In a preliminary report the cyc A gene has been mapped to within 100kb of the photosynthetic gene cluster of Rb. sphaeroides on the R' plasmid pWS2 (Kiley and Kaplan, 1988).

1.5. REGULATION OF PHOTOSYNTHETIC MEMBRANE ASSEMBLY

1.5.1. ASSEMBLY OF THE PHOTOSYNTHETIC APPARATUS

The photosynthetic apparatus of Rb.sphaeroides is localised within the ICM systen, which although it is continuous with the CM, is structurally distinct (Niederman and Gibson,1978). The ICM can be separated from the CM by disrupting the membrane, giving rise to uniformly closed and photosynthetically competent vesicles called chromatophores (Niederman and Gibson, 1978). In photoheterotrophically grown cells the chromatophore fraction can comprise up to 30% to 50% of the total cell protein (Fraley et al., 1978), most of which is the light harvesting and reaction centre proteins (Clayton and Haselkorn, 1972; Broglie et al., 1980).

When chromatophores are prepared from cells undergoing pigment induction by a switch from growth in high to low oxygen, a less dense pigmented band is apparent when the membranes are separated on a sucrose density gradient (Niederman et al., 1976; Niederman et al., 1979). This band contains reaction centre, LH1 and LH2 complexes. Pulse chase experiments show that newly synthesised reaction centre and LH1 complexes appear in this fraction before it assumes the sedimentation characteristics of the ICM fraction. Some LH2 is present in this fraction, but a small proportion is not fully coupled for energy transfer to the LH1/reaction centre core (Hunter et al., 1979a) indicating that this fraction may be the site of formation of the invaginations of the CM, eventually becoming mature ICM. Labelling kinetics for the appearance of pigment protein complexes in the membrane indicate that this upper pigmented band fraction is the site of formation of the membrane invagination and is formed by insertion of LH1 and reaction centre complexes into the CM (Niederman et al., 1981).

The synthesis of bchl and phospholipid has been studied in Rb. sphaeroides undergoing a shift from high to low oxygen

growth conditions. It appears that the synthesis of bchl is localised in this transient upper pigmented band, rather than the ICM (Hunter et al., 1982), but that the site of phospholipid biosynthesis is in discrete areas of the CM (Radcliffe and Niederman 1984; Radcliffe et al., 1985). This adds further credence to the idea that the upper pigmented band which is the site of insertion of pigment protein complexes grows through the addition of pigment protein complexes and not phospholipid.

Low temperature spectral analysis of this upper pigmented band and the ICM fractions during pigment induction indicate that the upper pigmented band is photosynthetically competent, with a high efficiency of energy transfer from bchl 850 to bchl 875 (Hunter et al., 1985). At the start of the induction the photosynthetic unit consists of several reaction centres surrounded by about 200 bchl 875 molecules, which by the end of the induction has increased to about 3000 bchl molecules. This large increase in the amount of bchl molecules in each invagination is due mostly to the insertion of LH2 in the ICM, since by the end of the induction there has been just a three-fold increase from about 50 to about 150 light harvesting bchl molecules per reaction centre. Thus the development of the ICM appears to involve the addition of LH2 in increasingly random aggregates around a highly ordered LH1/reaction centre core.

The kinetics of light driven electron flow in the upper pigmented band indicated reduced levels of the Rieske iron-sulphur protein of the ubiquinol-cytochrome C_2 oxido-reductase with respect to levels found in mature ICM (Bowyer et al., 1985), indicating that this fraction is not totally competent for light driven, cyclic electron flow.

The effect of light on the synthesis of the photosynthetic apparatus has also been studied. In a rather abrupt transition Rb.sphaeroides has been transferred from chemoheterotrophic growth in the dark with high aeration to

anaerobic photoheterotrophic growth (Chory et al., 1984). All growth ceased after this induction, as well accumulation of cell protein, DNA and phospholipid. Cell growth did not restart for a further 12 hours. Bchl, LH1 and LH2 synthesis was found to start after about 2 hours, the LH1 and LH2 being inserted into the ICM in a stepwise fashion. Small indentations were found from freeze/fracture electron microscopy of cells at the start of the induction, which may represent the transient sites of insertion of pigment protein complexes into the CM. The problem with this study is that it involved the effect of two variables, light and oxygen, and it is not clear how important each factor is.

The effect of shifting Rb.sphaeroides from high light to low light anaerobic growth conditions has also been studied (Hunter et al., 1982; Farelly and Niederman, 1987). In the latter study cells were subjected to a nearly 20 fold reduction in light intensity from 32,000 to 1,800 lux. This time cell growth only ceased for about 1 hour, and within 3 hours the bchl content had risen 2 fold reaching a peak at 6 hours. LH1 only increased 1.5-fold in this time, whereas LH2 had increased 4.5-fold reaching a peak after 6 to 9 hours of a 6-fold increase. These results show that the ICM is expanded upon reduction of light intensity largely by the increased addition of LH2 into the ICM. The upper pigmented fraction is present throughout this induction indicating that in these conditions this region is still the site of synthesis of the ICM.

Supporting evidence for this transient upper pigmented band being the site of ICM comes from observations of the internal membrane structure of mutants lacking LH2; in electron micrographs the membranes have the morphology of tubules rather than the characteristic vesicles (Hunter et al., 1988b; Kiley et al., 1988). Addition of LH2 to the growing photosynthetic unit may be necessary for forming the spherical invaginated structure since the curvature may be

imposed on the membrane by aggregation of LH2 subunits (C.N. Hunter, personal communication).

When cell division in synchronous cell populations occurs there must be a mechanism whereby the number of ICM vesicles is kept constant in each daughter cell. It has been suggested that in view of the change in the density of the ICM during cell division the ICM vesicles are replicated, partly by the insertion of extra phopholipid (Fraley et al., 1979). A mechanism for coupling the division of the ICM with cell division is difficult to envisage, and this fluctuation of density of the ICM may be due to the formation of new ICM vesicles after cell division from the transient and less dense upper pigmented band membrane fraction (Niederman et al., 1979), which could tend to reduce the density of the total photosynthetic membrane fraction.

1.5.2. REGULATION OF GENE EXPRESSION

1.5.2.1. PIGMENT BIOSYNTHESIS GENES

It has been known for a long time that the synthesis of bchl is regulated by oxygen (Cohen-Bazire et al., 1957) and that the level of activity for at least one of the biosynthetic enzymes, δ-aminolaevulinate synthase, is also affected by oxygen (Lascelles, 1975). The purification of most of the biosynthetic enzymes for bchl and carotenoid is very difficult and so in order to examine the effect of oxygen and light on the synthesis of bchl and carotenoid biosynthetic enzymes, it is necessary to use genetic techniques.

Biel and Marrs (1983) have examined the effect of oxygen on the expression of a number of cloned bch genes by constructing lac Z fusions with five different bch genes. Upon transferring aerobically growing cells from 20% to 2% oxygen, they found that the level of β galactosidase activity rose 2 to 4 fold.

The level and stability of mRNA coding for bchl and crt biosynthetic exzymes has been studied in Rb.capsulatus in response to oxygen and light (Clark et al., 1984; Zhu and Hearst, 1986; Zhu et al., 1986a). It was found that oxygen reduces the level of bch specific mRNA, but increases it for crt mRNA, and that more mRNA for bch and crt genes was present under low light growth conditions than for high light. Oxygen was also found to decrease the stability of mRNA. The increased level of crt mRNA with moderate levels of oxygen was thought to be involved in the protective function of carotenoids in scavenging oxygen radicals. The expression of bch E, B, A and C genes has been studied during the high to low oxygen pigment induction system of Niederman et al. (1976) in Rb.sphaeroides (Hunter and Coomber, 1988). The level of expression of the genes reached a peak 1 hour after the induction, at a point when the level of bchl had reached only 7% of the maximum level reached. Unfortunately all of the work described above on both Rb.capsulatus and Rb.sphaeroides which measures the levels of mRNA's for pigment biosynthesis used probes containing more than one gene, which would tend to obscure the effects on the expression of particular genes. The level of mRNA in cells gives an indication of the level of expression of genes, although this does not measure the rate of transcription, as the level of mRNA is a function of both synthesis and degradation, which may vary differently under different environmental conditions.

1.5.2.2. GENES FOR PIGMENT BINDING PROTEINS

The levels of mRNA coding for the pigment binding proteins are also affected by oxygen. This was first demonstrated by Clark et al. (1984) who showed that the level of mRNA from the puf operon increased nearly 40 fold when Rb.capsulatus cells are transferred from high oxygen to low oxygen growth conditions. More detailed analysis of transcripts from the puf operon of

Rb.capsulatus (Belasco et al., 1985) revealed the presence of three major transcripts of 2.7 kb, 0.5 kb and 0.49 kb. These transcripts had the same 5' end and therefore appeared to initiate from same point. The sizes indicated that the small transcripts could encode puf B and A and the large 2.7kb transcript puf B, A, L, M and X (see figure 9a), and would therefore comprise an operon. The ratio of the two small transcripts to the large transcript was approximately 10:1, which could account for the observed stoichiometry of LH1 complexes to reaction centre in the ICM (Kaufmann et al., 1982). The 3' ends of the small transcripts were mapped, and found to differ by between 4 to 14 nucleotides.

Northern hybridisation of RNA from Rb.sphaeroides revealed a similar pattern for the puf transcripts of 0.3 kb and 2.6 kb (Zhu and Kaplan, 1985) which was subsequently revised to 0.5 kb and 2.6 kb (Zhu et al., 1986b; Hunter et al., 1987). The two transcripts in Rb.sphaeroides were found in a similar ratio to that for Rb.capsulatus. These transcripts have also been found in Rhodospirillum rubrum (Bélanger and Gingras, 1988). Northern analysis of RNA probed with cloned DNA for the LHII genes has found that the polypeptides are encoded by a single 0.55kb message in Rb.capsulatus (Zhu and Hearst, 1986) and Rb.sphaeroides (Kiley and Kaplan, 1987; Hunter et al., 1987).

The effect of light and oxygen on these transcripts has been studied. The steady state levels of transcripts from Rb.sphaeroides for encoding LH1, reaction centre L and M apoproteins were found to be repressed by oxygen, and under photoheterotrophic conditions the levels of the transcripts was slightly higher under low light conditions relative to high light (Zhu and Kaplan, 1985). Similar results have been found for Rb.capsulatus, where the levels of mRNA encoding LH1,

LH2 and reaction centre L, M and H apoproteins have been studied (Zhu and Hearst, 1986; Donohue *et al.*, 1986a; Zhu *et al.*, 1986a). The reaction centre H gene appears to be regulated by light and oxygen in a similar way to the *puf* transcripts. The *puc* transcript was found to vary over a much larger range than the *puf* transcripts, reaching higher levels under low light anaerobic conditions, but being much more sensitive to the level of oxygen in the medium, and light. Similar results for *puc* have been found in *Rb.sphaeroides* (Kiley and Kaplan, 1987). By transferring *Rb.capsulatus* from photoheterotrophic growth to aerobic chemoheterotraphic growth, and inhibiting transcription with proflavine, the half lives for *puf*, *puc* and *puh* transcripts were found to be halved, indicating that oxygen also exerts some sort of postranscriptional control as well as regulating transcription (Zhu *et al.*, 1986a). Unfortunately one must question the chemoheterotrophic growth conditions used by Zhu *et al.*, 1986a) which included strong illumination, which may be toxic to the cell in combination with oxygen, thus making mRNA less stable.

When *Rb.sphaeroides* cells are transferred from very high aeration to low aeration growth conditions in the dark as described by Niederman *et al.*, (1976), pigmentation and assembly of the photosynthetic apparatus occurs in a well defined order under the influence of only one variable, the oxygen tension in the medium. The changes in levels of mRNA encoding LH2, LH1 and reaction centre L amd M apoproteins have been measured during this induction (Hunter *et al.*, 1987). The 2.7 kb and 0.5 kb *puf* operon transcripts reached a peak after 2 hours, whereas the 0.55 kb *puc* operon transcript reached a peak at 4 hours, matching the insertion of these complexes into the ICM. These observations are in agreement with the known order of assembly of the photosynthetic apparatus, which starts

with LH1/Reaction centre core complexes and continues by addition of LH2 (Niederman et al., 1976).

The origin of the two puf operon transcripts in Rb.capsulatus was analysed by performing pulse chase experiments, followed by inhibition of transcription (Belasco et al., 1985). Radioactivity first appeared in the 2.7 kb transcript, before disappearing from this transcript, and appearing in the 0.5 kb transcript. This would indicate that although these two transcripts have the same 5' ends, the 0.5 kb transcript appears to arise through 3' processing of the 2.7 kb transcript. The different half lives of the two transcripts would seem to support this (Belasco et al., 1985), the 0.5 kb transcript being much more stable with a half life of 22 minutes, compared to the half life of the less stable 2.7 kb transcript of 5 minutes. Two possible hairpin loop structures were found in between the puf A and puf L genes which correspond to the 3' ends of the 0.5 kb and 0.49 kb transcripts, which could act as the sites for RNA processing or terminators for 3' exoribonucleases, which may degrade the larger transcript.

Similar differences in the half lives of these two messages were found in Rb.sphaeroides (Zhu et al., 1986b). Oxygen was found to reduce the half lives of the transcripts by about a half. The 5' ends of the puf transcripts was measured and were found to be different. The large transcript started 75 nucleotides upstream of puf B, whereas the small transcript started 104 nucleotides upstream of puf B. These results would seem to indicate that the two transcripts arise in Rb.sphaeroides through initiation of different points, but they could also arise through 5' processing. The measured 5' end 104bp upstream of puf B of the 0.5kb transcript may only represent a fraction of the ends,

and the rest of the 5' ends of the small transcript may start at -75bp.

The effects of deleting the intercistronic hairpin loop from the puf operon of Rb.capsulatus was examined (Klug et al., 1987; Chen et al., 1988). The small 0.49/0.5 kb transcripts were undetectable, but there was no change in the level of the large 2.6 kb transcript, or its half life. The amount of LH1 complex relative to reaction centre was greatly reduced, and photosynthetic growth and light adaption in LH2 deficient mutants was severely impaired. Insertion of the intercistronic puf stem loop after puf X at the end of the puf operon failed to increase the stability of the large 2.6 kb transcript (Chen et al., 1988). The putative puc mRNA stem loop terminator was also placed just downstream of the puf stem loop, upstream of puf L. Two transcripts of 0.5 kb and 0.56 kb were found, the large 2.6kb transcript was undetectable. It appeared that the puc stem loop did act as a transcriptional terminator. The half life of the 0.56 kb transcript was 17 minutes, but the half life of the 0.5 kb transcript had almost doubled to 35 minutes. These results would appear to indicate that the puf stem loop acts as a terminator or inhibitor for 3' exoribonuclease action, rather than endonucleolytic cleavage at the stem loop. The large message is not stabilised by a stem loop being placed at its 3' end, presumably because 3' exoribonuclease action makes a cut upstream from the 3' end of the transcript, before progressing in the 5' direction towards the stem loop. The addition of a second stem loop (from puc) in between puf A and puf L doubles the stability of the 0.5kb message, presumably because 3' exoribonucleases have to get through two stem loop structures to degrade this message. In addition, as mentioned above there is a second effect of transcriptional termination which removes or decreases the amount of 2.6 kb transcript to start with.

Recent work on the 5' region of the puf operon of Rb.capsulatus has shown the presence of another gene called puf Q (Bauer et al., 1988). They showed that this open reading frame is transcribed from two promoters as part of the puf operon, 699 and 511 bp upstream of puf B. These promoters were mapped by performing interposon mutations of lac Z fusions with puf Q and puf M and by 5' mapping. Transcription from the -699bp promoter was found to be regulated by oxygen in a similar way to the puf transcripts, whereas the -511bp promoter was responsible for a low level constituitive expression, implying that there must be labile 3.2 and 3.0 kb transcripts. Transcription initiation from these sites was found not to require puf encoded gene products. The puf operon transcripts must be rapidly processed to the 2.6 kb and 0.5 kb transcripts to a point some 100 bp upstream of puf B, as the 2.6 kb transcript is the largest message detected by Northern analysis. It is not clear whether the puf operon transcript in Rb.sphaeroides could arise in the same way, as the large and small transcripts have been found to have different 5' ends (Zhu et al., 1986b). In Rb.sphaeroides a mechanism linking 5' and 3' processing, or a totally different mechanism involving transcriptional initiation may operate if the mapping studies of Zhu et al., (1986b) are correct.

Upstream of puf B there is an open reading frame called ORF K, of 20 amino acids which has 9 rare codons (Kiley and Kaplan, 1988). This may be involved in some form of attenuation, possibly affecting the differential processing of the 5' ends of the small and large transcripts of Rb.sphaeroides

Post-transcriptional regulation of mRNA for LH2 in Rb.capsulatus has been shown to be important in light adaption of the photosynthetic apparatus (Zucconi and Beatty, 1988). They showed that the puc A and B genes

are transcribed on two mRNA's which have 5' ends about 125 and 110 nucleotides upstream of puf B, and identical 3' ends about 80 nucleotides downstream of puc A, present in similar quantities. It was not clear if these two messages arise by transcription from different promoters, or are formed at least in part by RNA processing. The level of mRNA in high light grown cells was 1.4 times that in low light grown cells, which when adjusted for the 3 times total RNA content of the high light grown cells, means there is 4 times as much puc mRNA in high light grown cells to low light grown cells. The half life of the transcript in low light was found to be twice that of the transcript in high light grown cells, which must reflect light dependant degradation of puc transcripts. In order to account for the observed levels of mRNA a far greater rate of transcription initiation must occur in the high light grown cells than in low light (possibly up to 8 fold greater). In order to account for the increased amount of LH2 complexes to the LH1/reaction centre core complex in the ICM of low light grown cells (Schumacher and Drews, 1978; Hunter et al., 1982; Farelly and Niederman, 1987), there must be a number of post-transcriptional controls affecting the level of LH2 complex found in the ICM, which in addition to mRNA stability which increases with decreasing light intensity, could include regulation of translation, regulation of the breakdown of nascent polypeptides and complexes, and availability of bacteriochlorophyll.

1.6. SUMMARY OF RESEARCH

The genes that encode the α and β polypeptides of the major light harvesting complex of Rhodobacter sphaeroides B800-850, have been cloned, mapped and sequenced through the use of oligonucleotides based upon the known polypeptide sequences. These genes, puc A and B, are transcribed in the order, B, A, are of 150 and 164 nucleotides respectively, and are separated by a spacer region of 15 nucleotides. Transfer of these genes to mutant M21 lacking the B800-850 complex has been accomplished and absorbance spectra of recombinant strains M2181 and M2184 show that expression of puc A and B is comparable to levels found in the wild type.

The relative levels of mRNA for the reaction-centre L and M subunits, B875(LH1) α and β polypeptides and B800-850 (LH2) α and β polypeptides, have been measured during pigment induction of Rhodobacter sphaeroides Over the 6 hours of the experiment, bacteriochlorophyll levels increased by at least 100-fold. No transcripts for photosynthetic components were detectable at the start of induction; after 2 h the levels of transcripts from the puf operon (encoding reaction-centre and B875 subunits) had reached the maximum; these transcripts were 2.7 and 0.5kb respectively. The transcript for the puc operon (B800-850 complex) was estimated to be 0.55kb and reached a maximum level after 6 hours. These results are consistent with the proposal that, during the assembly of the photosynthetic apparatus, the synthesis of B875 reaction-centre aggregates precedes that of the major antenna, B800-850.

An LH2$^-$ strain of Rb.sphaeroides was constructed by deleting the puc operon (which encodes the LH2 α and β polypeptides) from the chromosome, and replacing it was a kanamycin resistance gene. Southern blot analysis indicated that the 950 bp BamHI fragment which contains the puc operon (as indicated by sequence data) has been lost, and replaced by a 1.25 kb fragment encoding the Km^r gene. This mutant strain, DBC1, was found to lack the LH2 complex as shown by the loss of the characteristic absorbance bands at 800nm and 850 nm, moreover LH2 α and β polypeptides were

undetectable by SDS-PAGE. The LH2 phenotype was restored to DBC1 by the transfer of a 3.8 kb BscI fragment in pMA81 containing the puc operon; transconjugants possessed a normal LH2 absorbance spectrum, and LH2 α and β polypeptides.

Two mutant strains DPF1 and MDPF1 derived from wild type Rb.sphaeroides and mutant M21 respectively have been studied. Each of these has had a 4.8 kb PstI fragment containing the puf operon selectively removed by a site specific recombination procedure. Spectroscopic analysis of both of these mutants shows complete loss of all reaction centre and antenna pigments, SDS-PAGE showed that the polypeptides of the reaction centre, LH1 and LH2 antennae were undetectable, including those of reaction centre H and LH2 α and β which are not encoded by the puf operon. DNA sequencing of a 2,228 bp BamHI-KpnI fragment upstream of puf L indicates the presence of two open reading frames denoted puf R and puf Q of 110 and 77 amino acids respectively and overlapping by 4 nucleotides. Northern analysis of RNA prepared from wild type Rb.sphaeroides has identified 5 hitherto unrecognised transcripts at the 5' end of the puf operon, 3 of which could encode puf Q and one which could additionally encode puf R. The 5' ends of these transcripts have been mapped by mung bean nuclease analysis to 748 bp and 690 bp upstream of puf B. Two regions of DNA have been found which are homologous with the Rb.capsulatus puf 1 and puf 2 promoters. The deduced amino acid sequence of puf Q and puf R show homology to the puf Q and a putative puf R gene from Rb.capsulatus.

An LH1$^-$ strain of Rb.sphaeroides was constructed by filling in then blunt end ligating an AccI site in the pufB gene. This manipulation caused a 2 basepair frame shift mutation in pufB which was presumed to result in a truncated LH1 polypeptide of 25 amino acids, reduced from 49 amino acids. In addition LH1 was absent. The putative mutated construct was checked by restriction enzyme analysis and nucleotide sequencing. The mutant strain was shown to lack the LH1 complex and constituent α and β polypeptides by spectral analysis and SDS-PAGE respectively.

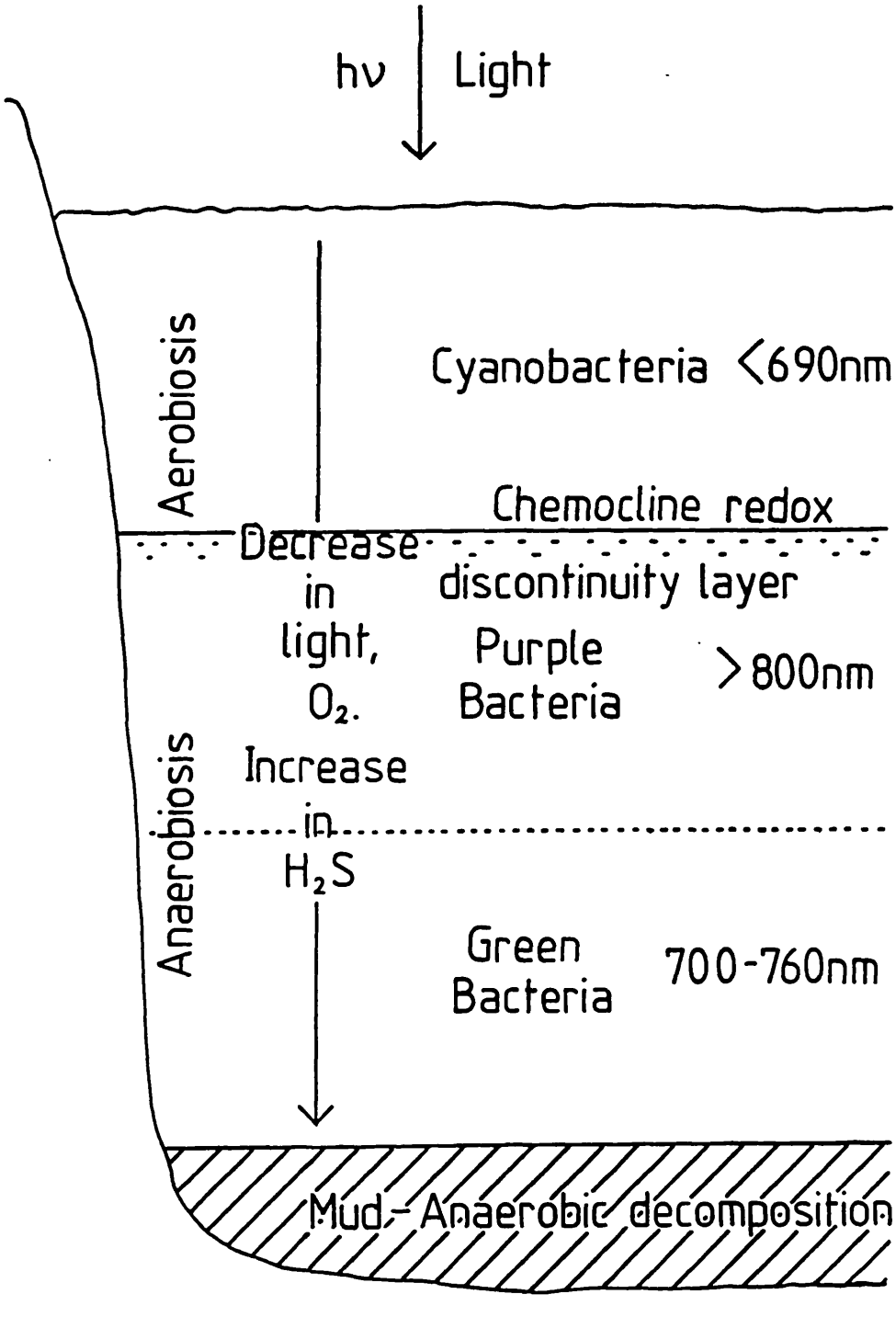
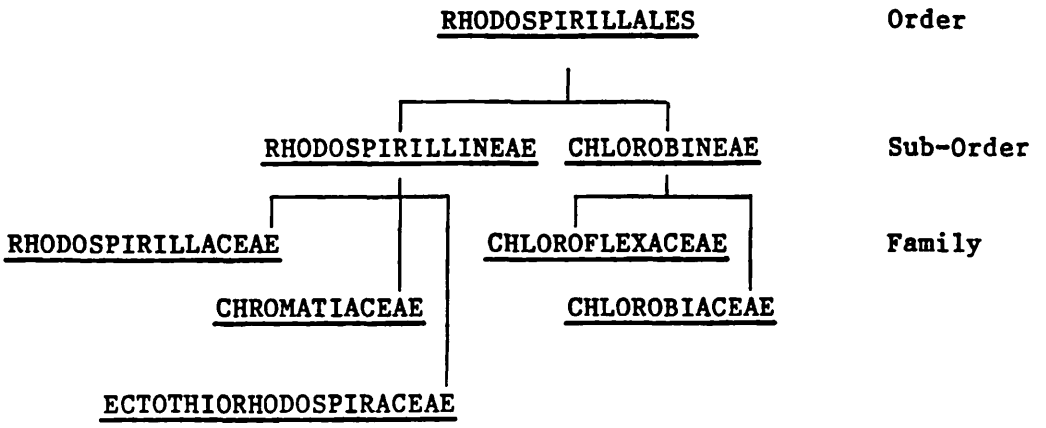


FIGURE 1.1.

Distribution and growth conditions for photosynthetic bacteria in a typical meromictic lake (Pfennig, 1978).



Genus and species of the family Rhodirillaceae:

Rhodospirillum	rubrum	Rhodopseudomonas	palustris
	fulvum		viridis
	molischianum		acidophila
	photometricum		blastica
	salexigens		
Rhodomicrobium	vannielii	Rhodopila	globiformis
Rhodobacter	capsulatus	Rhodocyclus	purpureus
	sphaeroides		gelatinos
	sulfidophilus		tenuis
	adriaticus		

Figure 1.2.

Classification of the Photosynthetic Bacteria (after Pfennig and Truper, 1984; Imhoff et al., 1984).

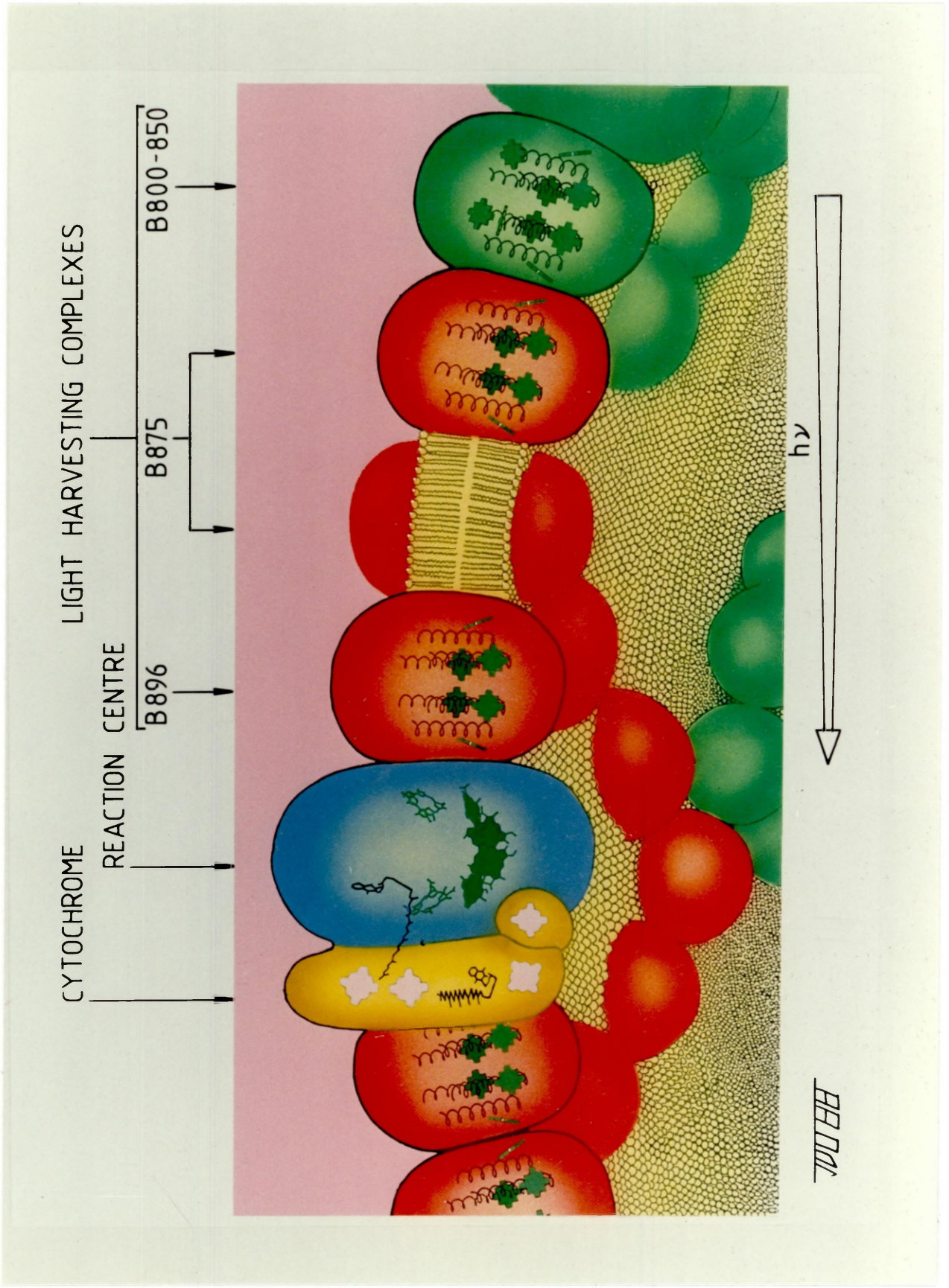


Figure 1.3. Schematic representation of the Photosynthetic Apparatus of Rb.sphaeroides. Artwork by J. Olsen.

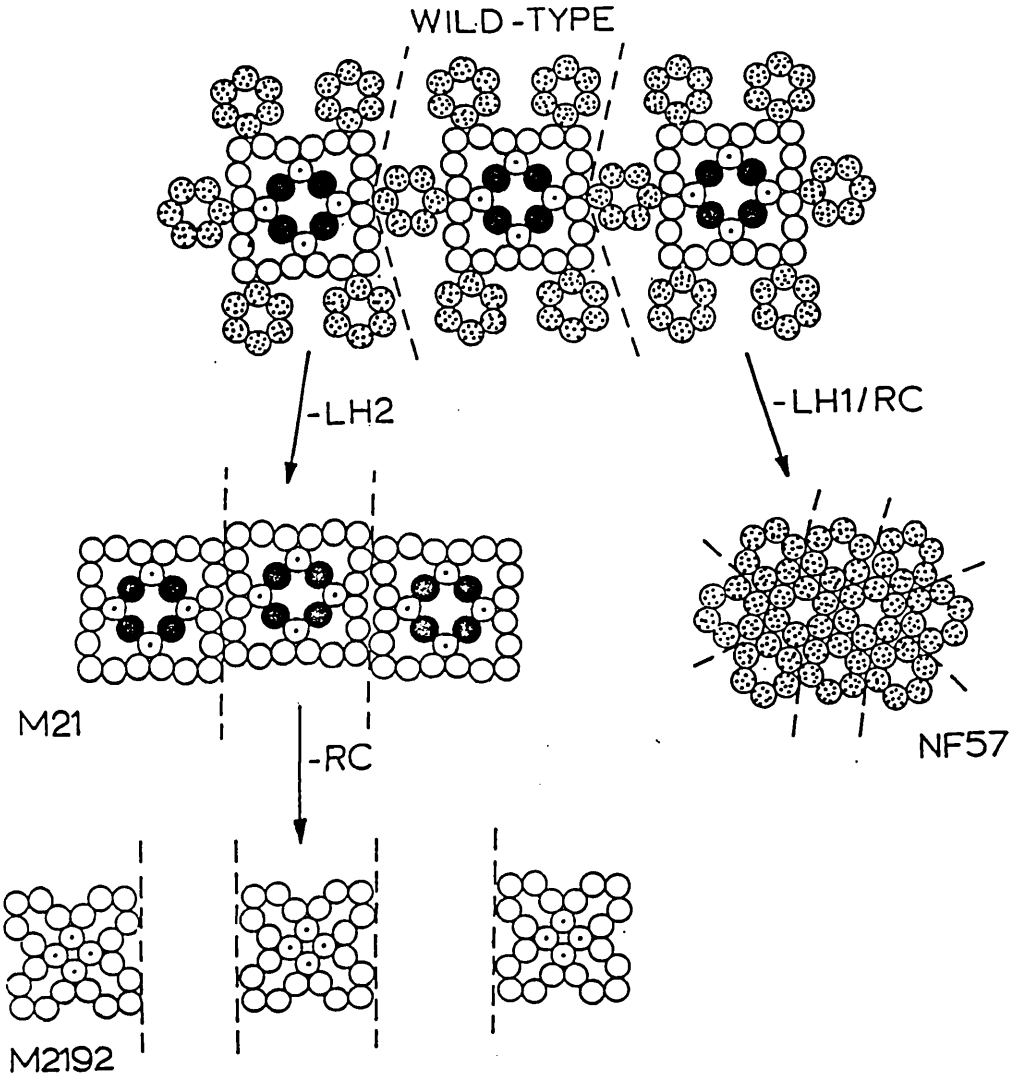


FIGURE 1.4.

Models for the arrangement of antenna and reaction centre complexes from wild type and mutant strains of Rb.sphaeroides. The dashed lines indicate domains at 4K (Hunter and van Grondelle, 1988).

⊛	LH2	- 6 bchl 850	3bchl 800
○	LH1	- 6 bchl 875	
⊙	LH1	- 6 bchl 896	
●	reaction centre	- 4 bchl	

FIGURE 1.5.

Primary structures of the α and β antenna polypeptides of the B870-890, B1017 and B800/850 antenna complexes from purple photosynthetic bacteria (Rhodospirillaceae). C: hydrophobic, polar, or charged cluster regions. (Zuber H., 1987)

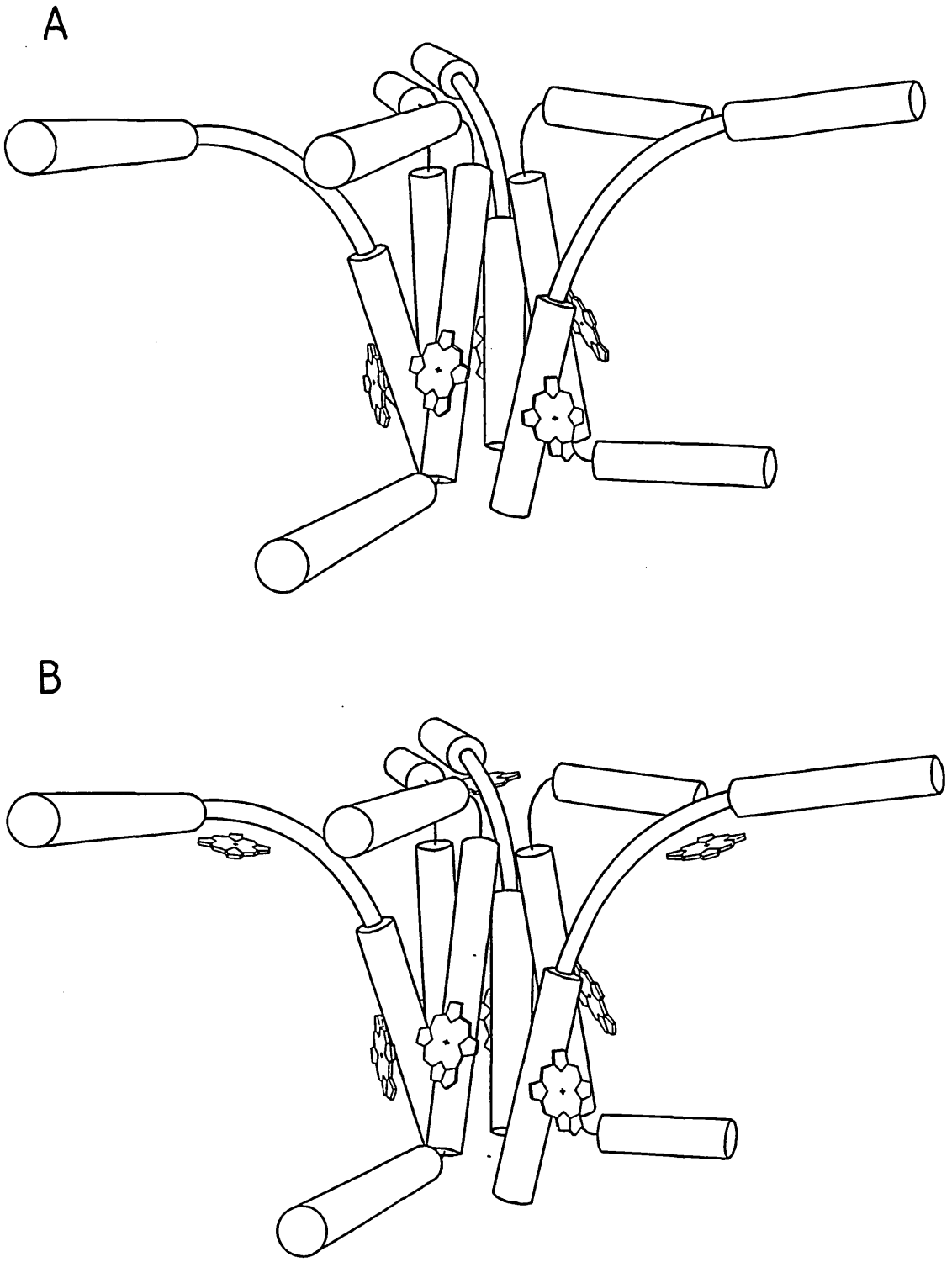
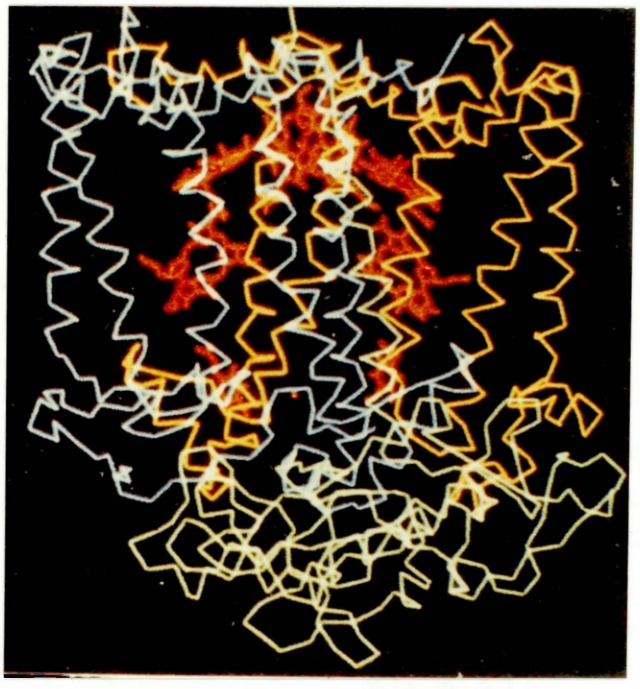


FIGURE 1.6.

Schematic representation of LH1(a) and LH2(b) $\alpha\beta$ trimer of Rb.sphaeroides. Structures predicted by Robson-Garnier computer prediction (Garnier et al., 1978). Data interpreted and artwork by J.D. Olsen. Polypeptide conformation: Line-random coil; small cylinder-coil; large cylinder-αhelix.

a



b

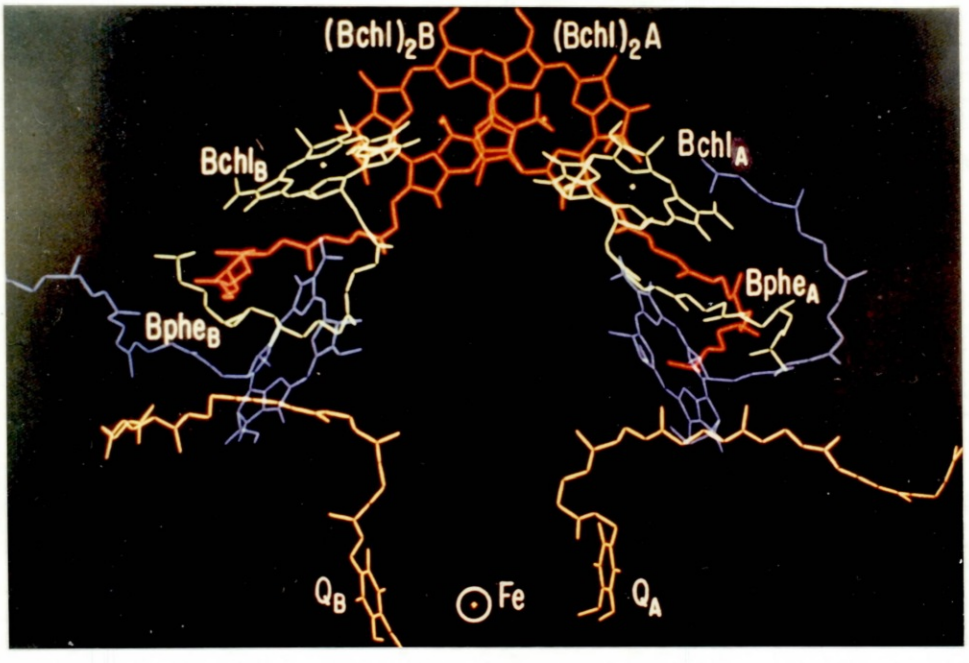


FIGURE 1.7.

View of Rb.sphaeroides reaction centre. a.) Polypeptide Subunits: L-yellow: M-blue, H-green. Cofactors-red. Allen et al. (1987a). b.) Structure of the cofactors. Allen et al. (1987b). Periplasmic side of the membrane is at the top, and the cytoplasmic side is at the bottom.

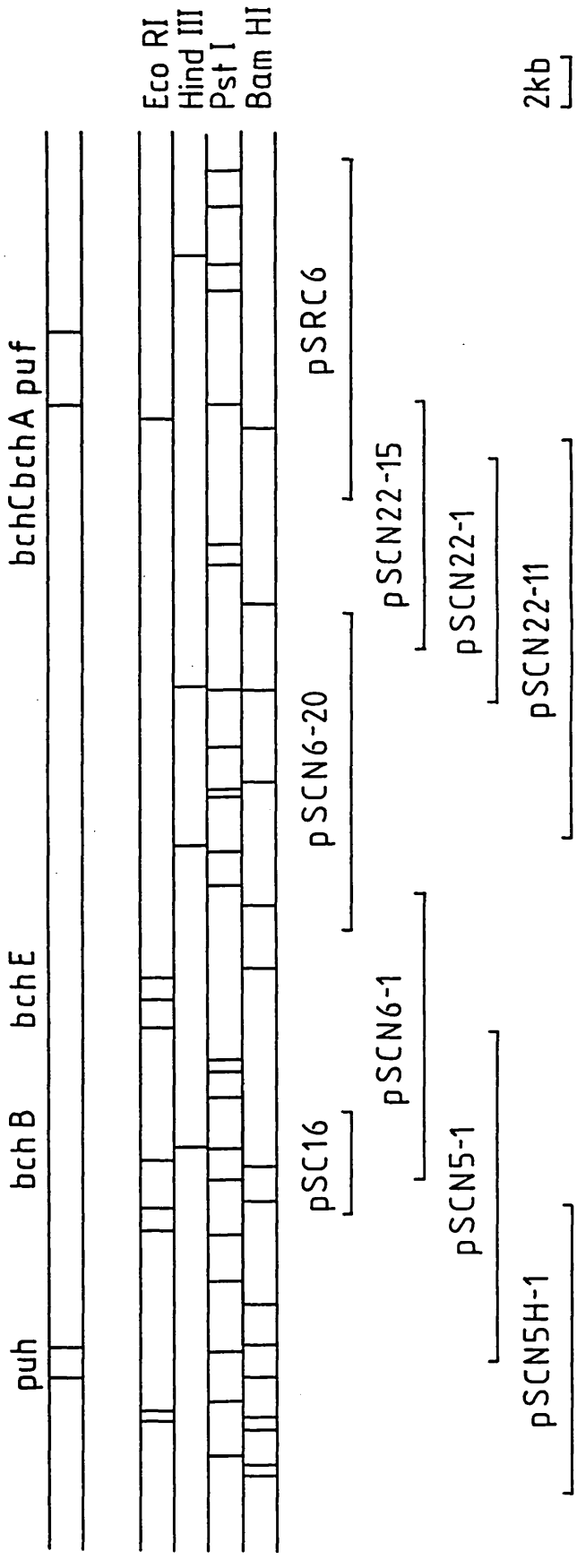


FIGURE 1.8.

Genetic map of photosynthetic gene cluster of Rb.sphaeroides alligned with restriction map (Coomber, S.A., personal communication).

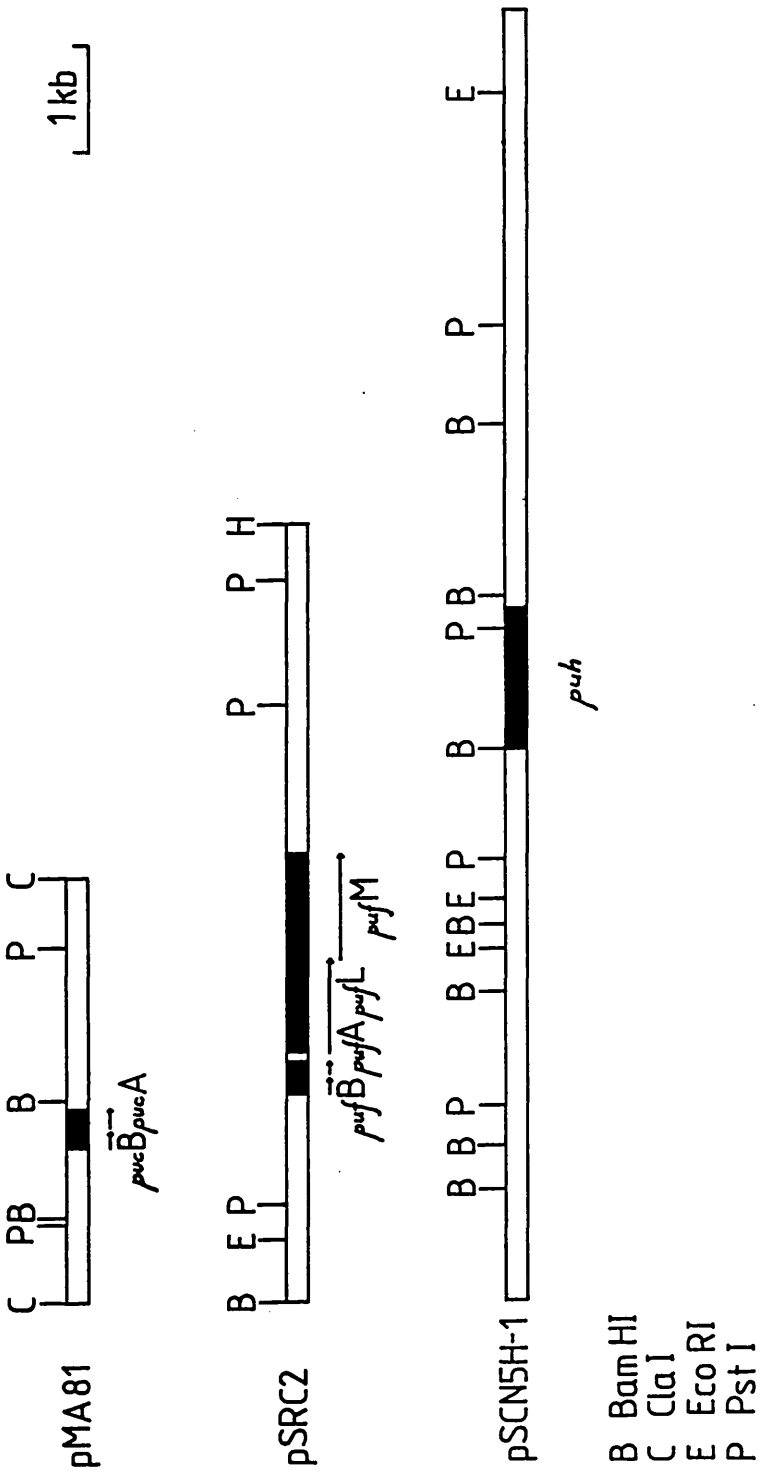


FIGURE 1.9.

Restriction maps of Rb.sphaeroides DNA. Only the inserts are shown. pMA81 (chapter 3). pSRC2 and pSCN5H-1 (Coomber S.A., personal communication).

TABLE 1.1.

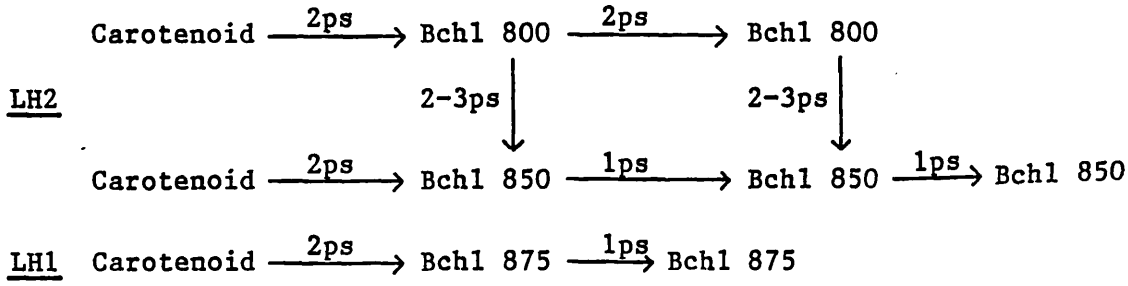
Domain Sizes of LH1 and LH2 Complexes of Rb.sphaeroides. The numbers for LH2 refer to Bchl 850 pigments (Hunter and van Grondelle, 1988).

	LH1		LH2	
	298K	4K	298K	4K
Membranes				
Wild-type	$\geqslant$1000	$\geqslant$100		45
M21	$\geqslant$ 330	100	-	
M2192	150	125	-	
NF57	-		$\geqslant$ 365	30
LDS- LH1	8		-	
LDS- LH2	-		30	
LDAO/Triton LH2	-		600	

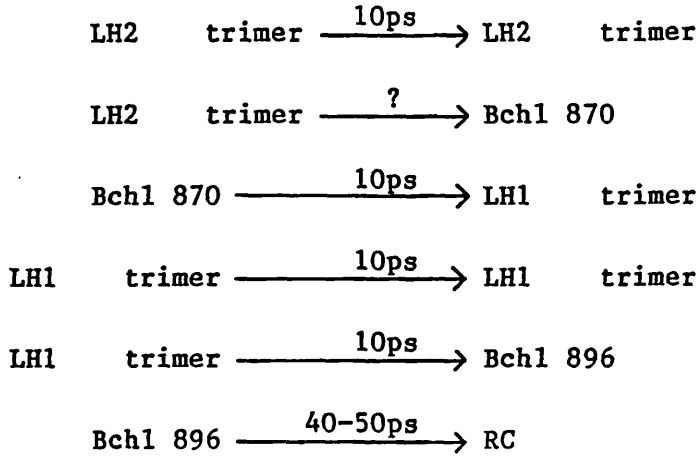
TABLE 1.2.

KINETICS OF ENERGY TRANSFER WITHIN THE PHOTOSYNTHETIC MEMBRANE (Hunter et al., 1988a).

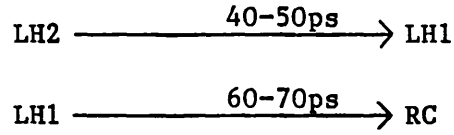
'Local' times within $\alpha\beta$ trimer pigment protein units



Transfer times between $\alpha\beta$ pigment protein units



Transfer times between pigment pools



CHAPTER 2

MATERIALS AND METHODS

2.1. SOLUTIONS AND MEDIA

The preparations of common solutions and *E.coli* growth media unless described in the text is as indicated in Maniatis *et al.* (1982). Bacterial growth media was prepared using reverse-osmosis water obtained using the Milli-RO 15 water purification system. Unless otherwise stated, nutrient broth and nutrient agar was used for *E.coli* growth. Analar grade reagents and solvents were used for the preparation of solutions mostly purchased from BDH Chemicals Ltd. All solutions were prepared using water obtained from the passage of reverse-osmosis water through the nanopure deionisation system from Barnsted.

Rb.sphaeroides was grown in M22+ medium, sometimes supplemented with 0.1% Casamino acids (see table 1). M22+ agar plates were poured with agar at a concentration 1.5 grams per 100 mls. Culture media and the majority of solutions were sterilised by autoclaving at 15 p.s.i. for at least 20 minutes or by filtration through sterile 0.2 μm disposable Nucleopore filters.

Thermolabile substances such as vitamins and antibiotics were not added to autoclaved solutions until they had cooled down below 55°C.

2.2. BACTERIAL STRAINS

E.coli and *Rb.sphaeroides* strains are listed in table 2. Bacteria were maintained on selective plates and stored at 4°C or as frozen stocks in 25% (v/v) glycerol in LB medium at -80°C for long term storage.

2.3. PLASMIDS

The plasmids used in this thesis are listed in table 3. Plasmid DNA was stored dissolved in TE at - 20°C.

2.4. MICROBIAL TECHNIQUES

2.4.1. Growth of E.coli

E.coli nutrient agar plates were incubated overnight at 37°C. E.coli cultures were grown in nutrient broth overnight at 37°C shaken at 200 r.p.m.

2.4.2. Growth of Rb.sphaeroides

Rb.sphaeroides was grown either aerobically, or photosynthetically without oxygen. Aerobic M22+ agar plates were incubated in the dark at 32°C for 3 to 5 days, whereas photosynthetic plates were placed in BBL anaerobic gas jars and illuminated by 40W tungsten bulbs, with fans maintaining the temperature at or around 30°C. Cells were cultured aerobically in M22+/0.1% Casamino acids at 32°C for 1 to 4 days shaking at 200 r.p.m. Photosynthetic cultures were grown in full, sealed bottles with no air present and illuminated as above.

2.4.3. Plasmid Transfer

Plasmids were transferred from E.coli Sm10 and S17-1 to Rb.sphaeroides by conjugal transfer as described by Hunter and Turner (1988). Kanamycin resistant plasmids were transformed into S17-1 for plasmid transfer; streptomycin and ampicillin resistant plasmids were transformed into Sm10 for plasmid transfer. A fresh Rb.sphaeroides culture was mixed at a ratio of about 1:50 with the E.coli donor and incubated on a dry LB agar plate for 4 - 8 hours before being resuspended in M22+; serial dilutions were spread on M22+ agar with the appropriate selection (antibiotic and/or photosynthetic) for tranconjugant Rb.sphaeroides.
The aerobic culture of Rb.sphaeroides for plasmid transfer was grown overnight from a single colony.

2.4.4. Antibiotic Concentrations

The concentrations of antibiotics for E.coli and Rb.sphaeroides cultures and selection are indicated in table 4.

2.4.5. Induction of Bacteriochlorphyll Biosynthesis

Pigment biosynthesis was induced in Rb.sphaeroides as

described by Niederman et al. (1976), in which a highly aerated non-pigmented culture of Rb.sphaeroides is transferred to oxygen limited growth conditions. The highly aerated non-pigmented culture is prepared by inoculating M22+ medium supplemented with 0.1% Casamino acids with 10mls of fresh culture of Rb.sphaeroides in M22+, such that the volume of media takes up less than 20% of the total volume of the flask. The culture is incubated at 32°C shaking at 250 r.p.m. When the absorbance at 680nm (A680) has reached 0.5 to 1.0 pigment biosynthesis is induced by harvesting the cells in sterile 250ml bottles at 10,000 r.p.m. and resuspending the cells in fresh M22+,0.1% Casamino acids such that the A680 is 2, and the volume of medium in the flask is 80% of the total volume. The culture is incubated in the dark at 32°C, shaking at 180 r.p.m.

Cells are harvested at times from 0 to 12 hours for analysis of bchl content, RNA or pigment protein content.

2.5. DNA MANIPULATION

2.5.1. Preparation of Plasmid DNA

Plasmids were prepared using E.coli DH5 as the host, and appropriate antibiotic selection for the plasmid. Small scale preparations (minipreps) were grown overnight in 10mls of nutrient broth and large scale preparations (maxipreps) were grown overnight in 500 or 1,000mls of nutrient broth. The room temperature cleared lysate method was used to isolate the plasmid (Hunter and Turner, 1988). Large scale plasmid preparations were further purified by centrifugation to equilibrium in caesium chloride-ethidium bromide gradients (Maniatis et al., 1982).

2.5.2. Preparation of Genomic DNA

Total DNA was prepared from Rb.sphaeroides lysed with lysozyme and sarkosyl and purified on a caesium chloride-ethidium bromide gradient as described by Hunter and Turner (1988).

2.5.3. Preparation of Competent Cells

E.coli cells competent for transformation by plasmid DNA were prepared by treating log-phase growing cells with rubidium chloride as described in 'Protocol 3' by Hanahan (1985). Aliquots of cells, 50-300µl in RF2 buffer, were stored at -80°C until required. Competencies of greater than 0.5% were obtained

E.coli JM105 cells competent for transformation by M13 and Bluescribe DNA were prepared by a modification of the calcium chloride procedure of Maniatis et al. (1982). 2ml of an overnight culture of JM105 in 2xTY (see section 2.5.15.) was used to innoculate 100ml 2xTY and the suspension was shaken at 160 r.p.m. until an A680 of 1.0 was obtained (2 to 3 hours). Cells were harvested by centrifugation at 3,000 r.p.m. at 4°C and resuspended in 50ml ice cold 50 mM $CaCl_2$. The cells were incubated on ice for 30 minutes and then repelleted and suspended in 10ml of ice cold 50mM Cacl ,20% (v/v) glycerol. The cells were dispensed into 300µl aliquots, and stored at -80°C.
Competencies of greater than 0.01% were obtained

2.5.4. Transformation of Competent Cells

Competent cells were transformed by plasmid DNA as described by Hanahan (1985) in 'Protocol 3'. DNA solution was mixed with at least 10x the volume of the thawed cell suspension. After outgrowth, the bacterial suspension was plated out on nutrient agar with appropriate antibiotic selection for the plasmid and then incubated overnight at 37°C. DNA solution containing up to 100ng/µl

2.5.5. Restriction Enzyme Digestion

Digestions of DNA with restriction endonucleases were usually carried out using the buffers recommended by the manufacturer or the low, medium or high salt buffers described by Maniatis et al. (1982). In addition 4mM spermidine, 100µg/ml BSA was added to the reaction as an aid to digestion. Digestion reactions contained no more than 200ng DNA/µl and 2 - 10 units of enzyme per µg DNA, the volume of enzyme comprising less than 10% of the total

volume. Reactions were usually carried out at 37°C and incubated for at least 3 hours, but usually overnight.

Partial digests were sometimes required, and were performed by either using a series of restriction enzyme concentrations 0.01-1.0 unit/µg DNA or by varying the time for digestion, 5 - 60 minutes. Reactions were stopped by placing the samples on ice while the degree of digestion was checked by agarose gel electrophoresis.

2.5.6. Ligation of DNA Fragments

Ligations were performed overnight at room temperature using T4 DNA ligase and the polyethylene glycol buffer described by King and Blakesley (1986). Typical ligation reactions contained 10 to 100ng of DNA per µl, with a 1 to 4-fold excess of insert over vector.

2.5.7. Blunt Ending of DNA with 5' Overhangs

DNA with 5' overhangs, generated by restriction endonuclease digestion was filled in by incubating 50 to 500ng of DNA in a total volume of 10 µl with a 1mM solution of dATP, dTTP, dCTP and dGTP and one unit of Klenow at 37°C for 1 hour.

2.5.8. Dephosphorylation of DNA

The 5' ends of cut vector DNA were dephosphorylated using calf intestinal phosphatase from BCL. DNA in a volume between 10 to 100 µl was digested with 0.5 µl of enzyme (12 units) at 37°C for 30 minutes. The enzyme was inactivated by the addition of 0.1 vol. of EGTA (0.2M, pH7.3) and heated at 65°C for 10 minutes, followed by phenol/chloroform (1:1 v/v) extraction and ethanol precipitation of DNA. Control reactions were performed to assay the efficiency of dephosphorylation Vectors for subcloning DNA fragments cut with two restriction enzymes were agarose gel purified (see section 2.5.11.) to remove the linker, rather than being dephosphorylated.

2.5.9. ^{32}P-Labelling of DNA

Radioactive ^{32}P-labelled DNA probes for Southern, Northern and colony hybridisation experiments were prepared by one of two methods.

Nick translation of DNA; The Amersham nick translation kit was used as directed by the manufacturers instructions (Rigby et al., 1977).

Mixed oligonucleotide fragment labelling; The method of Feinberg and Vogelstein (1983) was used whereby mixed oligonucleotides are used as primers for transcription of the denatured DNA fragment. This method was found to give much higher incorporation of radioactive label into the DNA fragment (about 90%) than nick translation, and was therefore used in preference.

The radiolabel [alpha $^{-32}$P]dCTP(3000Ci/mmole;10mCi/ml) was purchased from New England Nuclear as the tetra (triethyl ammonium) salt. Routinely 1 µl (10 µCi) was used to label 50-125ng of DNA. Unincorporated radionucleotides were removed by passage through a 1ml column of Sephadex G50 (purchased from Pharmacia) equilibrated in TE as described by Maniatis et al. (1982).

Oligonucleotides were labelled with [gamma $^{-32}$P]-dATP as described by Woods (1984). The radiolabel was purchased from New England Nuclear.

2.5.10. Agarose Gel Electrophoresis

Routine DNA analysis was carried using agarose gels made from ultra-pure electrophoresis-grade agarose purchased from BRL. A tris-borate-EDTA (TBE) buffer containing 0.5µg/ml of ethidium bromide was used to prepare the gels and as the running buffer. Gels were run in Bio-rad horizontal gel apparatus with the current and voltage not exceeding 100mA and 100V respectively. DNA bands were visualised under 254nm UV light by comparing their electrophoretic mobility

with that of DNA fragments of known size, normally BglI or HindIII digests of lambda DNA. Gels were normally photographed with a Polaroid camera carrying an orange filter and using Polaroid Type 667 black and white instant film. 5 x loading buffer (0.25%[w/v] bromophenol blue, 25% [w/v] Ficol-type 400 in water) was added to DNA samples to help in their application to the wells and as an indication of how far the DNA had migrated. The concentration of agarose in the gel was adjusted depending on the size of DNA fragments being analysed as described by Maniatis et al. (1982). Plasmid restriction enzyme digests were run for 1 to 5 hours. Genomic DNA restriction enzyme digests for Southern blotting were run on gels overnight at 10 to 20 volts.

2.5.11. Purification of DNA Fragments from Agarose Gels

Electrophoresis was performed in a gel made from SeaKem HGT agarose purchased from FMC Bioproducts. The DNA fragments were purified by electroelution into dialysis bags as described by Maniatis et al., (1982). The protocol used differed in a number of respects. The dialysis bag storage buffer additionally contained 1mM sodium azide. The eluted fragment was removed from the dialysis bag, and the gel fragment washed with a further 50-100µl of TE. The eluted fragment and washing were combined and then directly extracted with phenol/chloroform(1:1v/v) and then chloroform. The DNA fragment was precipitated with ethanol/sodium acetate and the pellet washed with 80% EtOH/H_2O. The pellet was dried in vacuo and dissolved in TE at an estimated concentration of 5 to 100 ng/µl. The purity and yield of fragment was checked by agarose gel electrophoresis.

2.5.12. Southern Blotting

The protocol for transfer of DNA from agarose gels to nitrocellulose is as described by Maniatis et al. (1982). The nitrocellulose was purchased from Schleicher and Schuell and had a pore size of 0.45µm.

2.5.13. Lysed Colony Filters

Bacterial colonies from agar plates were transferred onto nitrocellulose filters for screening as described by Woods (1984). In some cases colonies were toothpicked onto sterile nitrocellulose on appropriate agar plates, the toothpicks then allowed to grow up; overnight for E.coli and over 2 to 4 days for Rb.sphaeroides. These toothpick filters were then lysed and washed in the same way.

2.5.14. Southern and Colony Hybridisations

Southern blot and lysed colony filters were bybridised under exactly the same conditions.

Hybridisation with ^{32}P-labelled DNA: This was performed essentially as described by Maniatis et al. (1982). The filters were prehybridised with hybridisation solution (6xSSC, 5xDenhardts, 0.5%SDS, 25% dextran sulphate, 100µg/ml denatured herring sperm or calf thymus DNA). The denatured ^{32}P-labelled probe was added directly to the bag 2 to 5 hours later without changing the hybridisation buffer. Radioactive Filters were washed as follows:-

3 x 5'	2 x SSC	0.1%SDS	Room temperature
2 x 30'	1 x SSC	0.1%SDS	65° C
2 x 30'	0.2 x SSC	0.1%SDS	65° C
1 x 30'	0.1 x SSC	0.1%SDS	65° C

Prior to exposure of the radioactive filter to X-ray film, the nitrocellulose filter was sealed in a heat sealable bag.

Hybridisation with ^{32}P-labelled oligonucleotide: This was performed exactly as described by Woods (1982). The radioactive nitrocellulose filter was sealed while still damp in a heat sealable bag prior to autoradiographic exposure. Radioactive nitrocellulose filters were often washed at higher stringencies after autoradiographic exposure, and then re-exposed.

2.5.15. Preparation of Single Stranded DNA

DNA fragments for sequencing and S1 analysis were ligated

into appropriately cut replicative form M13 mp18 or mp19 (Norrander et al.,1983), which was purchased from Pharmacia. The ligations were transformed into competent E.coli JM105 cells.

JM105 was streaked out on minimal-glucose plates (per litre) NH_4Cl 0.8g; $Na_2SO_4.12H_2O$,4.5g; K_2HPO_4,3g; KH_2PO_4,1g; Agar, 15g; supplemented with glucose (2g) and thiamine (5mg) to maintain selection for the F' of JM105. Transformation of competent JM105 cells and purification of single stranded DNA and solution used is essentially as described by Bankier et al. (1987), but modified as follows:
0.3ml of competent JM105 cells were mixed with up to 10µl of a ligation mix and incubated on ice for 40 minutes, and then heat shocked at 42°C for 90 seconds and returned to ice. To 3ml of top-H agar (kept molten at 50°C) was added 25µl X-Gal (40mg/ml in dimethyl formanide), 10µl IPTG (100mg/ml in water) and the transformed cells. This mixture was quickly swirled and poured onto TYE plates.

The diluted overnight JM105 culture was inoculated with phage using 10µl phage stock, or by removing a white recombinant plaque from a plate with a blue Gilson pipette tip and blowing the agar plug into the medium. The culture was shaken vigorously at 37°C for 6 hours. The culture was transferred to a 1.5ml Eppendorf tube and the cells pelleted by spinning for 10 minutes in a microfuge (12,000g). 1ml of supernatant was transferred to a fresh tube taking care not to disturb the pellet. The remaining 0.5ml containing the cell pellet was mixed with 0.5ml LB/glycerol (50% glycerol in LB v/v) to make a phage stock and stored at -20°C. The supernatant was mixed with 250µl of PEG solution and incubated at room temperature for 5 minutes. The phage particles were pelletted by spinning for 10 minutes in a micro-fuge and the supernatant allowed to drain off for 5 minutes. The tubes were spun for a further 30 seconds to bring down any PEG solution from the sides of the tube, which was then removed using a fine pipette tip. The phage

pellet was suspended in 400µl TE. 200µl of phenol/chloroform was added and the tubes were briefly vortexed 5 times and then left at room temperature for 10 minutes. The tubes were then vortexed as before and left again for 10 minutes. The tubes were centrifuged for 10 minutes in a microfuge. 340µl of the supernatant was carefully removed which was extracted again with phenol chloroform. 270µl of supernatant was removed extracted with chloroform, and then 200µl of the supernatant was removed and single stranded DNA precipitated with ethanol and sodium acetate. The single stranded DNA was dissolved in 20µl of TE and 2µl of this was run on a fresh 1% agarose gel against M13 mp18 or mp19 as a molecular weight standard to check the yield, purify and the presence of an insert in the single stranded clone.

2.5.16. Sequencing of Single Stranded DNA

The dideoxy chain termination method of Sanger et al.(1977) was used to determine nucleotide sequences. The protocol and solutions used are essentially as described by Bankier et al.(1987), with the following details and modifications:

a.) the nucleotide analogue 7-deazo dGTP was substituted for dGTP to reduce secondary structure partly caused by the high GC content of Rb.sphaeroides DNA (Kaplan, S. personal communication).

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

c.) the elongation reactions were carried out at 50°C to reduce secondary structure formation caused by the high GC contents of Rb.sphaeroides DNA. For this reason after 15 minutes of the sequencing reaction a further 0.2 Units of Klenow fragment was added to each well, and the reaction allowed to proceed for a further 15 minutes. Klenow (0.2 units per well) was also added to the chase dNTPs.

d.) The large, un-notched glass plate was treated with a 0.4% solution of silane and the notched plate with Sigmacote. When the plates were separated following electrophoresis the gel stuck to the large un-notched plate. Following fixation of the gel in acetic acid, the gel and plate were rinsed with water, carefully patted dry with Kleenex tissues and dried for 2 to 3 hours at 80°C. The X-ray film was placed in direct contact with the dried gel, inserted into a light tight bag, and left for 1 to 3 days at room temperature.

2.6. RNA MANIPULATION

2.6.1. Preparation of RNA

Total RNA was prepared from Rb.sphaeroides as described by Hunter and Turner (1988).

2.6.2. Northern Analysis of RNA

RNA was analysed on formaldehyde denaturing gels as described by Maniatis et al.(1982) containing 1.5% agarose. The BRL RNA ladder was used as molecular weight standards. The gel was run and blotted as described by Maniatis et al. (1982). Hybridisation of the blot with ^{32}P labelled DNA probe was as described by Thomas (1983).

2.6.3. Quantification of Signals on Autoradiographs

Signals on the autoradiographs of Northern blots were quantified using densitometry with a Shimadzu C5-930 scanner.

2.6.4. 5' end Mapping of RNA

Mung bean and S1 nuclease analysis was performed as described by Maniatis (1982). ^{35}S labelled single-stranded DNA was prepared by incubating primed M13 mp18 and mp19 recombinant clones with 1mM dCTP, dTTP and 7-deazo dGTP, 1 unit of Klenow and 8µCi deoxyadenosine 5'-(alpha-[^{35}S]) thio-triphosphate at 50°C for 20 minutes, and chased for 15 minutes as described for sequencing. DNA and RNA were

hybridised together for 3 hours at 55°C and digested in 30mM sodium acetate (pH4.6), 50mM NaCl, 1mM ZnCl, 5% glycerol at 37°C with mung bean or S1 nuclease for 2 hours. The DNA/RNA hybrid was precipitated with ethanol, and dissolved in 4µl TE. The sample was denatured with formamide buffer as described for DNA sequencing and subjected to electrophoresis on a denaturing 4% polyacrylamide gel with a DNA sequencing ladder as molecular weight standards.

2.7. PROTEIN MANIPULATION

2.7.1. Spectroscopy

Room temperature absorbance spectra were obtained using a Perkin-Elmer 554 spectrophotometer. Whole cells were suspended in a small amount of water and then mixed with a large volume of glycerol to minimise light scattering. Membrane preparations were diluted with water so that the maximum absorbance reading being scanned was less than 2.5.

2.7.2. Preparation of Membranes

Rb.sphaeroides wild type and mutant strains were grown in M22+ supplemented with 0.1% Casamino acids. Membranes were prepared from harvested cells by the method described by Hunter & Turner (1988).

2.7.3. SDS-PAGE

Isolated membranes were analysed on acrylamide gels using the buffer system described by Schumacher and Drews (1978), and a 12-20% gradient of acrylamide.
Gels also contained 50mM NaCl (Cogdell,R.J. personal communication).Membrane samples were treated with 3xLaemmli buffer(SDS 9%,B-mercaptoethanol 15%, tris HCl pH 6.8 187mM)for 8 minutes at 55'C.

TABLE 2.1.

M22+ medium (Hunter and Turner, 1988)

	Amount per litre of medium
KH_2PO_4	3.06g
K_2HPO_4	3.00g
Potassium Lactate	1 or 2g
$(NH_4)_2SO_4$	0.5g
NaCl	0.5g
Potassium succinate	4.6g
Sodium glutamate	0.27g
Aspartic acid	0.04g
Solution C	20mls

up to 1 litre with deionised water, pH7.6

Solution C	Per Litre
Nitriloacetic acid	10g
$MgCl_2.6H_2O$	24g
$CaCl_2.2H_2O$	3.34g
EDTA	125mg
$ZnCl_2$	261mg
$FeCl_2.4H_2O$	251mg
$MnCl_2$	90mg
$(NH_4)_6Mo_7O_{24}.4H_2O$	9.25mg
$CuCl_2.2H_2O$	7.8mg
$Co(NO_3)_2.6H_2O$	12.4mg
H_3BO_4	5.7mg

1000 x Vitamin supplement. Add to M22+ after autoclaving

	Per Litre
Nicotinic acid	1g
Thiamine	0.5g
Para-aminobenzoic acid	0.1g
Biotin	10mg

TABLE 2.2.

BACTERIAL STAINS

Strains	Genotype	Source or Reference
E.coli		
DH5	derivative of DH1 that is: F^-, *end* A1, *hsd* R17 (rk^-mk^+), *sup* E44, *thi*$^-$1, λ^-, *rec* A1, *gyr* A96	Hanahan (1985)
JM105	del (*lac-pro* AB), *supE*, *thi*, *strA*, *endA*, [F' *traD36*, pro A B, *lacI*^{q} Z del MI5]	Cesareni *et al.*, (1985)
Sm10	RP4-2-Tc::Mu. Km^r, *Thi*, *Thr*, *Leu*, su_{III}	Simon *et al.* (1983)
S17-1	RP4-2-Tc::Mu Km::Tn7 Tp Sm^r, *Pro*, res^-, mod^+	Simon *et al.* (1983)
Rb.sphaeroides		
NCIB 8253	Wild type	R.A. Niederman, Rutgers University
M21	$LH2^-$	Ashby *et al.* (1987)
M21.81	Sm^r	Ashby *et al.* (1987)
DBC1	$LH2^-$ del (*puc-BamHI*) Km^r	This thesis
DBC1.81	Sm^r Km^r	This thesis
DPF1	Psg^- del (*puf-PstI*) Km^r	This thesis
DPF1(pSRC6)	Wild type	This thesis
MDPF1	$LH2^-$ Psg^- del (*puf-PstI*) Km^r	This thesis
MDPF1(pSRC6)	$LH2^-$	This thesis
MA3	$LH1^-$	This thesis

TABLE 2.3.

PLASMIDS

Plasmid	Description	Source or Reference
pAT153	Amp^r, Tet^r	Twigg and Sherratt (1980)
pNH_2	Sm^r Tc^r derivative of RSF1010; 8.05kb EcoRI-AvaI fragment of RSF1010 ligated to EcoRI-AvaI fragment of pAT153 bearing the Tc^r gene	Hunter and Turner (1988)
Bluescribe M13+/M13-	Amp^r derivative of pEMBL with polylinker restriction site block from pUC-18, and M13 intergenic region	Purchased from Northumbria Biolabs Ltd.
M13 mp18 & mp19	Derivative of M13 with lac ZY and polylinker restriction site block	Purchased from Pharmacia
pSUP202	Amp^r , Cm^r , Tet^r , mob.	Simon et al .(1983)
pJW2	13kb BamH1 fragment of Rb.sphaeroides 2.4.1.DNA cloned in pBR322, containing the puf operon	Williams et al .(1983)

TABLE 2.4.

Concentration of antibiotics in μg/ml.(Hunter and Turner,1988;Hunter and Coomber,1988) Carbenicillin,M.K.Ashby(data not shown).Antibiotic concentrations sufficient for at least 99.99% kill.

Antibiotic	E.coli	Rb.sphaeroides
Ampicillin	50	-
Carbenicillin	-	1
Chloramphenicol	25	-
Kanamycin	30	20
Nalidixic acid	30	-
Neomycin	30	30
Streptomycin	25	50
Tetracycline	10	-

CHAPTER 3

CLONING, NUCLEOTIDE SEQUENCE AND TRANSFER OF GENES FOR THE B800-850 LIGHT HARVESTING COMPLEX OF RHODOBACTER SPHAEROIDES

3.1. INTRODUCTION

The light-harvesting antenna of the photosynthetic bacterium Rhodobacter sphaeroides consists of the major complex, B800-850, arranged around a cluster formed by B875 antenna and approximately four reaction centres (Vos et al., 1986). Within the photosynthetic membrane, approximately 1000 bacteriochlorophyll molecules are connected to form a domain for efficient energy transfer (van Grondelle et al., 1983; Hunter et al., 1985) and excitation energy is eventually trapped by a reaction centre where the primary photochemical event occurs.

The bacteriochlorophyll and carotenoid pigment of B800-850 and B875 are non-covalently bound to small polypeptides designated α and β . These simple antennae have been extensively characterised (Kramer et al., 1984b: Drews, 1985; Zuber, 1985) and they are therefore attractive model systems for further studies involving manipulation of antenna structure through mutagenesis of pucA and B genes.

Here, the genes encoding the B800-850 α and β polypeptides, pucA and B, have been cloned and sequenced. These genes have been transferred to mutant M21 which lacks the B800-850 antenna, resulting in the restoration of a wild-type light-harvesting system.

3.2. RESULTS

A mixed 23-base oligonucleotide deduced from the published amino-terminal sequence of the α subunit of the B800-850 complex (Theiler et al., 1984) was made on an Applied Biosystems 380B synthesiser using methyl phosphoamidite, and is shown below:

```
N-MET-THR-ASN-GLY-LYS-ILE-TRP-LEU-C
5-ATG-ACA-AAC-GGA-AAA-ATA-TGG-TC-3'
       G   T   G   G   C     C
       C       C       T
       T       T
```

This oligonucleotide has a degeneracy of 384. A 17 base mixed

oligonucleotide was also made, but corresponding to the opposite DNA strand.

An Rb.sphaeroides gene bank was used which had been prepared by ligating DNA partially digested with TaqI into the ClaI site of pNH_2 (Hunter and Turner, 1988). The bank contained about 8000 colonies in E.coli DH5, had an average insert size of 5.5kb, where f =0.999 (Maniatis et al., 1982). 6,000 colonies of the gene library were probed with the ^{32}P labelled 23-base oligonucleotide. Postively hybridising colonies were toothpicked and rescreened. Four positively hybridising clones were obtained and were used to prepare plasmid DNA (pMA81-4). Digestion with EcoRI and HindIII show that pMA81 has an insert of 3.8kb and pMA82,83 and 84 inserts of about 7kb (data not shown). Southern blot analysis of restriction enzyme digests of these plasmids probed with the oligonucleotide show that these plasmids contain single bands which hybridise to the oligonucleotide including small PstI and BamHI fragments (figure 1a). The restriction patterns of pMA82, 83 and 84 were identical (data not shown). Detailed restriction mapping and Southern blot analysis was performed on pMA81 and an example is shown in figure 1b. A 950bp BamHI fragment of pMA81 was found which contained at least part of the puc A gene (figure 2).

This fragment was subcloned into M13 mp18 for preliminary sequencing. The puc A gene was identified by comparison of the deduced polypeptide sequence with the amino acid sequence determined by Theiler et al. (1984). Sequencing of the 1,078 bp XhoI - PvuII fragment (see figure 2) was completed by sequencing further along the BamHI fragment in M13 using the 17-base oligonucletide as a sequencing primer, and sequencing the XhoI - PvuII fragment in both orientation in M13 mp18 and mp19 and is shown in figure 3.

Two open reading frames were found which encoded polypeptides in agreement with the sequence of the published α and β polypeptides of Theiler et al. (1984) with the exception of an exchange of isoleucine for leucine at position 37 in the β chain of the

sequence reported here. This difference is not expected to have any effects on the conformation or function of the polypeptide (Theiler, R., personal communication), and I attribute this to a difference between the wild type strain (NCIB 8253) that I have used, and that used by Theiler (2.4.1.) As has already been found for Rb.capsulatus (Youvan and Ismail, 1985) the genes are transcribed in the order puc B, A and are of 150 and 164 nucleotides respectively. Each gene is preceded by a possible site for ribosome binding, and the genes are separated by a spacer region of 15 nucleotides.

In order to isolate an $LH2^-$ mutant a frozen stock of Rb.sphaeroides which had been previously treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was used (Hunter and Turner, 1988). A 100 fold dilution of the frozen stock of NTG treated Rb.sphaeroides was plated out on M22+ plates. The plates were incubated aerobically for 3 days at 32°C until colonies had just started to appear. The plates were transferred to anaerobic gas jars and grown photosynthetically for a further 6 days. A total of 32 colonies were toothpicked which appeared to be less pigmented and some were smaller in size than wild type Rb.sphaeroides colonies. Aereobic cultures of these mutants were grown and the cells analysed spectrally. One mutant, M21 was found, which lacked the characteristic LH2 absorbance (see figure 4.a). This mutant has been characterised by spectroscopy at 4K (van Dorssen et al., 1988) and the amount of B800-850 complex present has been determined by circular dicroism to be less than 1% (van Dorssen et al., 1988). SDS-PAGE of membranes isolated from M21 indicate that the α and β polypeptides of the LH2 complex are absent (Hunter and van Grondelle, 1988). Northern analysis of RNA prepared from M21 cannot detect puc mRNA (data not shown).

Mutant M21 was restored to LH2 phenotype by conjugal transfer of pMA81 containing the puc operon, mobilised from E.coli Sm10 (see figure 4.b.)

The absorbance spectra in figure 4a shows M21, with peaks at 875nm due to LH1 and a small peak at 800nm due to the reaction centre. Transconjugant strain M2181 (figure 4b) has a spectrum nearly identical to that of wild type Rb.sphaeroides (figure 4c), with characteristic absorbance peaks at 800 nm and 850 nm due to LH2 and a shoulder at 875nm due to LH1.

3.3. DISCUSSION

The genes encoding the α and β polypeptides of the LH2 complex of Rhodobacter sphaeroides have been cloned and found to be transcribed in the order puc B, A as has been found in Rb.capsulatus (Youvan and Ismail, 1985). The nucleotide sequence is almost identical to the sequence of the puc A and B genes from the Rb.sphaeroides strain 2.4.1. (Kiley and Kaplan, 1987). The puc B and A genes are encoded on a single transcript of about 550bp (see chapter 4, Kiley and Kaplan 1987) and would therefore be transcribed together and so constitute a single operon. End mapping of the puc transcript in Rb.capsulatus has shown that the mRNA originates about 120bp upstream of puc B and ends about 80bp downstream of puc A (Zucconi and Beatty, 1988). There are no open reading frames within the region spanned by the transcript as mapped by Zucconi and Beatty (1988) which indicates that the 550bp puc transcript only encodes the puc B and A genes.

The genetic lesion in M21 resulting in loss of LH2 has not been determined, but is complementable by the 3.8 kb BscI fragment of pMA81 encoding the puc operon. Therefore the mutation which may be in the structural or regulatory sequences, and appears to result in loss or reduced levels of puc mRNA, is present within this 3.8kb BscI fragment which contains the puc operon in the chromosome of M21. Since its initial isolation and characterisation mutant M21 has been used to study energy transfer (Vos et al., 1988; van Dorssen et al., 1988; Hunter and van Grondelle, 1988; van Grondelle et al., 1988) and the size of energy transfer domains (Vos et al., 1988; Hunter and van Grondelle, 1988) in the photosynthetic apparatus of Rb.sphaeroides which has been discussed in the introduction to this thesis. Electron microscopy of M21 show that the membrane

invaginations are organised into tubular structures rather than vesicles (Hunter et al., 1988b) indicating that the LH2 complex plays an important role in the formation of the mature intracytoplasmic membrane vesicles (Niederman et al., 1979).

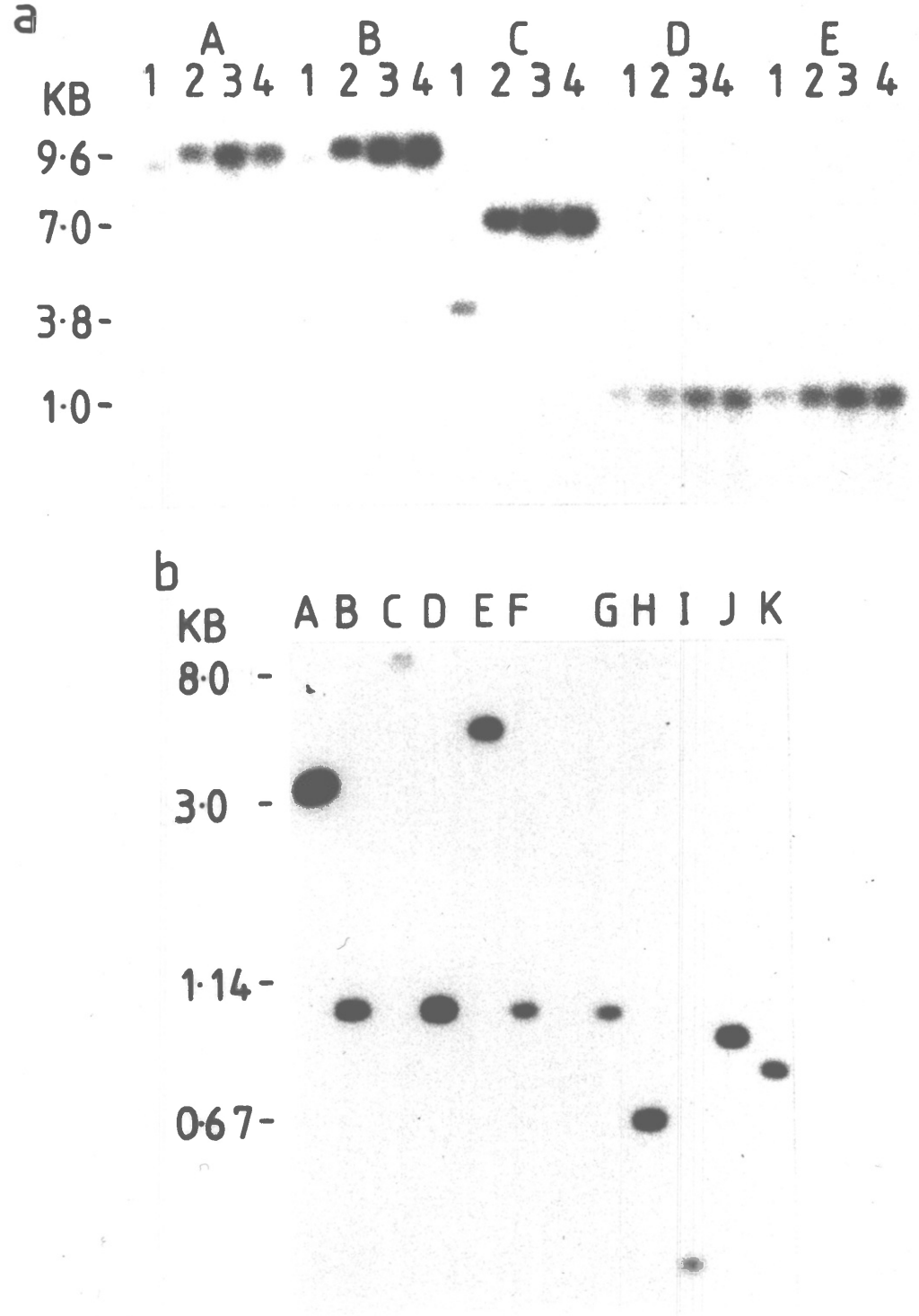


FIGURE 3.1.

Southern blot analysis of recombinant clones probed with ^{32}P labelled 23 base oligonucleotide. Sizes shown in kilobases.
Figure 1.a. 1-pMA81; 2-pMA82; 3-pMA83; 4-pMA84. digested with A-EcoRI; B-HindIII; C-EcoRI-HindIII; D-BamHI E·BamHIEcoRI.
Figure 1.b. pMA81 digested with A-BscI; B-BscI-BamHI; C-KpnI; D-KpnI-BamHI; E-HpaI; F-HpaI-BamHI; G-BamHI-SmaI; H-TaqI; I-TaqI-BamHI; J-AvaI; K-AvaI-BamHI.

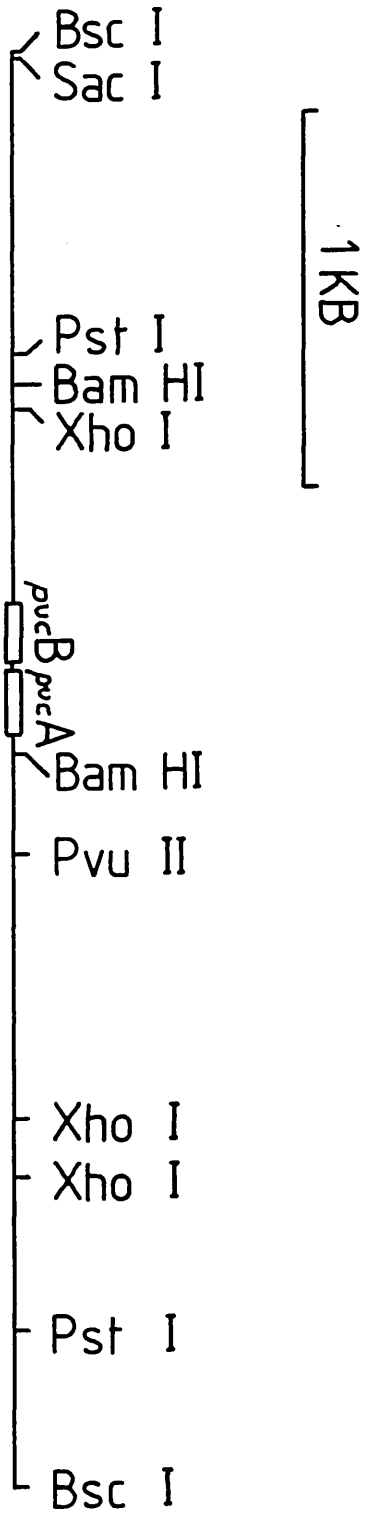


FIGURE 3.2.

Restriction map of the 3.8kb Bsc I fragment from pMA81 containing the puc operon.

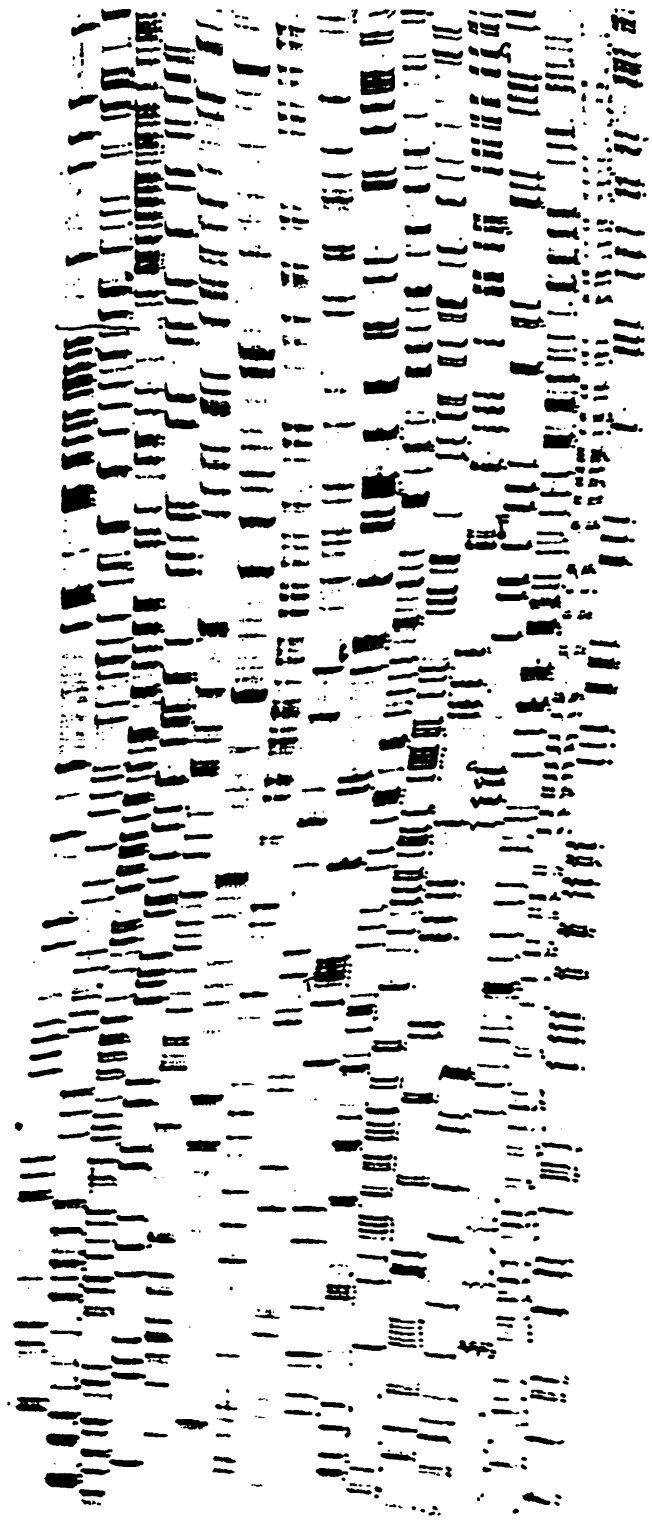


Figure 3.2a. Example of autoradiograph of sequencing gel used for puc operon sequencing.Tracks left to right T,C,G,A. Samples left to right BamHI,BamHI primed with puc oligonucleotide, mp18 XhoI-PvuII, mp19 XhoI-PvuII.

```
        10                  30                  50
CTCGAGCGGTTCGAGTCGGTGCTCGCGGGCCCTGTCGATAGTAGCGGGGCTGTATTGAGG

        70                  90                  110
CAGAGGCCGCCAAGCCATCCTGAAATCTCGAAAAATCCCCGCAAGATGGGGTCCGGAAGG

       130                 150                 170
GCCGGGCGCAAGGCCTCGGACACCCTCGTTTTTGCAGCAGCGAGAGGCTGCGGGACGGCC

       190                 210                 230
CTGTGGGGCCGGGACACGCAGCGTCAATTTCCCGCGCGCCTGCGGCAAAATTGTCCCTTT

       250                 270                 290
TCAAGCCGTTACGCAGGATTCCCGGCCGATCTGGCGGCCAATAAGTCGCACCCAAAACGG

       310                 330                 350
CCTTGTCAGCCAACAGTGACATTGAATCTGTCAGCGCAATGTGACACCCATAATGCGAGC

       370                 390                 410
CGGGGCGGATCAGAAATCGCCGACAAGGTGATCCAGGTCTCTCCGGTCTCGTCGAAGCCC

       430                 450                 470
GCGTGCAGGCCCTACACGCAAACCGTCGATTTACCAGTTGGGAGACGACACAATGACTGA
                                              pucB  MetThrAs

       490                 510                 530
CGATCTGAACAAAGTCTGGCCGAGCGGCCTGACCGTTGCCGAAGCCGAAGAAGTTCATAA
pAspLeuAsnLysValTrpProSerGlyLeuThrValAlaGluAlaGluGluValHisLy

       550                 570                 590
GCAACTCATCCTCGGCACCCGCGTCTTCGGTGGCATGGCGCTCATCGCGCACTTCCTCGC
sGlnLeuIleLeuGlyThrArgValPheGlyGlyMetAlaLeuIleAlaHisPheLeuAl

       610                 630                 650
CGCCGCTGCGACCCCGTGGCTCGGCTGATAGGAGAAGACTGACATGACCAACGGCAAAAT
aAlaAlaAlaThrProTrpLeuGly            pucA  MetThrAsnGlyLysIl

       670                 690                 710
CTGGCTCGTGGTGAAACCGACCGTCGGCGTTCCGCTGTTCCTCAGCGCTGCCGTCATCGC
eTrpLeuValValLysProThrValGlyValProLeuPheLeuSerAlaAlaValIleAl

       730                 750                 770
CTCCGTCGTTATCCACGCTGCTGTGCTGACGACCACCACCTGGCTGCCCGCCTACTACCA
aSerValValIleHisAlaAlaValLeuThrThrThrTrpLeuProAlaTyrTyrGl

       790                 810                 830
AGGCTCGGCTGCGGTCGCGGCCGAGTAATGCTGCGCAAGGCGCGGGCCTGCGGGCCCACG
nGlySerAlaAlaValAlaAlaGlu

       850                 870                 890
CCAGCCAGTCCGTGAGTTCCGAGCAGGCCGGGATCCTTGGGTTCCGGCTGCTCGTCACAC

       910                 930                 950
CAGCGACGTGTTCCTGTCGCCTTGCCCGTTCGGGCTGTGTGCCTGAAGCCCGAGATCCTT

       970                 990                1010
TCCAGTCGCGCAGTCTGCGCCTACCCCGGGATTCTTCACGCATGAGCCGAATTGCCGAAC

      1030                1050                1070
ATCTGGTCCGCATCGGACCGCGGTTTCTGCCATTTCCGACGCGGCCTCGGACCAGCTG
```

FIGURE 3.3.

Nucleotide sequence of the 1,078 XhoI-PvuII fragment showing the deduced amino acid sequence of the genes encoding B800-850 and polypeptides.

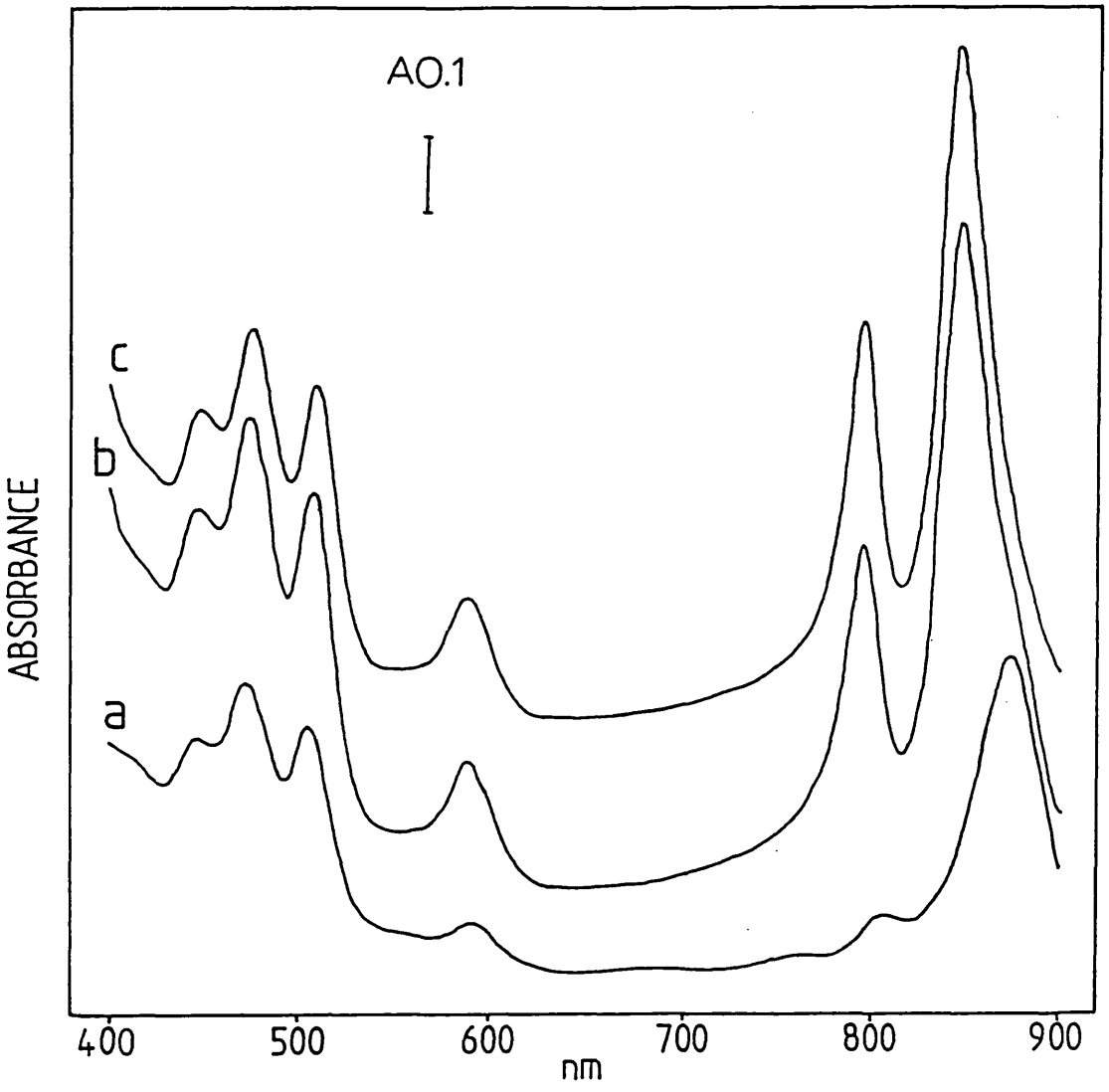


FIGURE 3.4.

Absorbance spectra of (a) mutant M21 and (b) transconjugant strain M2181, with (c) wild type containing cloning vector pNH2 included as a control. Absorbance spectra of whole cells.

CHAPTER 4

EFFECT OF OXYGEN ON LEVELS OF mRNA CODING FOR REACTION-CENTRE AND LIGHT-HARVESTING POLYPEPTIDES OF RHODOBACTER SPHAEROIDES

4.1. INTRODUCTION

The photosynthetic bacterium Rhodobacter sphaeroides provides an excellent model system for the study of photosynthetic membrane assembly, because the levels of photosynthetic components can be manipulated by light intensity or by oxygen tension. The photosynthetic apparatus is composed of three pigment proteins, the reaction centre, and light-harvesting antenna complexes B875 and B800-850. These pigment-protein complexes are absent in highly aerated cells, but when transferred to low-oxygen growth conditions, the complexes appear and are found to be located in invaginations of the cytoplasmic membrane. The synthesis of reaction centres and B875 antenna predominates in the early stages of photosynthetic unit assembly, whereas later stages are characterized by the addition of the B800-850 complex (Niederman et al., 1976).

The apoproteins for these complexes are encoded by the genes puf A, B, L and M (encoding B875 α and β polypeptides and reaction-centre L and M polypeptides respectively) and puc A and B (B800-850 α and β polypeptides respectively), with the puf and puc genes organized into an separate operons (Ashby et al., 1987; Zhu et al., 1986b; Kiley and Kaplan, 1987)).

Previous studies have reported that the ratio of B875 to reaction centres is relatively constant but that a variable amount of B800-850 antenna is present in the photosynthetic unit, depending on oxygen tension or light intensity (Niederman et al., 1976; Aagaard and Sistrom 1972; Holmes et al., 1980). The genetic basis for this differential response of the two antenna systems is unknown. The transcription of the puf operon has now been analysed in some detail (Belasco et al., 1985; Zhu et al., 1986b) but there is no information on the relative levels of transcripts for puf and puc genes. Now that the puc genes have been cloned (Ashby et al., 1987) it is possible to investigate the assembly

the assembly of all of the pigment-binding components of the photosynthetic apparatus using specific gene probes to estimate the levels of transcripts for reaction-centre B875 and B800-850 apoproteins. The system I have chosen to examine is that used by Niederman *et al.* (1976) in which a highly aerated non-pigmented suspension of *Rb.sphaeroides* is transferred to oxygen-limited growth conditions. This triggers the induction of photosynthetic membrane assembly over a period of up to 20h. RNA has been prepared from cells at various times after transfer to low-aeration growth conditions and used in hybridization analyses with cloned gene probes. I find that mRNA levels for reaction-centre and B875 apoprotein genes reach a maximum early on in development, whereas mRNA levels for B800-850 apoprotein genes exhibit a more gradual increase.

4.2. RESULTS

Figure 1 shows the induction of light-harvesting complexes in a low-aeration culture of *Rb.sphaeroides*; after 2h some pigmentation is apparent in the absorbance spectra of whole cells, with B875 present in nearly equal proportion to B800-850. Subsequently, the production of B800-850 outstrips that of B875; results similar to this have been analysed in more detail by Niederman *et al.* (1976).

The structure of the gene probes is shown in Figure 2. Figure 2(a) shows pJW1, which contains the B875 and reaction-centre genes, with the 0.51kb *Alu*I fragment (Zhu *et al.*, 1986b) which was used to assay mRNA encoding B875, pJW1 and a 1.2kb *Pvu*II-*Nru*I fragment specific for reaction centres have also been used. Figure 2(b) shows pMA81, the 950bp *Bam*H1 fragment and the location of the *puc* A and B genes. Their identity has been confirmed by DNA sequencing and comparison with the established amino acid sequence (Chapter 3; Ashby *et al.*, 1987).

Northern blots of *Rb.sphaeroides* RNA were prepared and hybridized with the probes described above. The time points chosen were clustered at the beginning of pigment induction because preliminary studies had indicated that, in the case of the *puf*

operon, there was a rapid early rise in the level of transcripts (Ashby et al., 1987b). From Figure 3(a) it is apparent that there are two major transcripts of 2.7 and 0.5kb plus three minor transcripts of 1.35, 0.85 and 0.65kb. This is in close agreement with Zhu et al. (1986b) who reported two major transcripts (2.6 and 0.5 kb) plus a minor transcript of about 1.45kb which is probably the 1.35 kb transcript reported here. The difference in the molecular mass of the two larger transcripts is not significant and may be due to the particular RNA markers used (with λ DNA markers the large transcript was 2.5 kb [Ashby et al., 1987b]) or the different methods used for isolation and gel electrophoresis of the RNA. Northern blots probed with a 1.2 kb PvuII-NruI fragment from the central coding region for the reaction centre L and M subunits show that the 2.7 kb transcript is the only mRNA species encoding the reaction centre L and M subunits (data not shown). The 0.51 kb AluI fragment from pJW1 which was used to probe this Northern blot contains the genes for the B875 α and β polypeptides plus about 200 base-pairs upstream (Zhu et al., 1986b). This fragment hybridizes to give transcripts including the large 2.7 kb band. These results show that, for the two major transcripts at least, the 0.5 kb message encodes only the B875 polypeptides, whereas the 2.7 kb message encodes both the reaction centre and B875 polypeptides which is in agreement with the work of Zhu and Kaplan (1985). The Northern blot also indicates that the level of the 0.5 kb B875 message is 10-20 fold higher than that of the 2.7 kb B875 reaction-centre message, which would account for the ratio of B875 antenna to reaction-centre complexes found in the photosynthetic apparatus (Niederman et al., 1976). More detailed analysis of the transcripts (Zhu et al., 1986b) has shown that their 5' ends lie 29 base-pairs apart, and that the difference in the levels of the two major transcripts arises from differential transcript initiation. The large puf transcript is referred to as 2.7kb as in most Northerns this transcript ran at this length, and is in closer agreement to Zhu et al. (1986b).

In order to investigate the transcription of puc genes, a Northern blot was probed with the 950 bp BamHI fragment of pMA81 (Figure 3b). It can be seen that the B800-850 α and

β polypeptides are encoded on a single transcript of approximately 0.55 kb.

The levels of the transcripts encoding the subunits of the photosynthetic complexes and the number of bacteriochlorophyll molecules per cell during the induction of pigment synthesis are shown in Figure 4; during the course of the experiment the amount of bacteriochlorophyll per cell increased at least 100-fold, broadly in line with the concentrations described previously (Hunter et al., 1985). The 2.7 kb transcript encoding reaction centres and B875 (Zhu et al., 1986b; Ashby et al., 1987b), rises fairly rapidly reaching a peak level after 2h, and then falls away. The level of 0.5 kb transcript encoding B875 only initially rises more gradually than that for the 2.7 kb transcript also reaching a peak at 2h, and then falls away more rapidly than the 2.7 kb transcript. The level of B800-850 transcript reaches a maximum after 6h, the level rising fairly rapidly between 1 and 2h from 10% to 85% of maximum.

4.3. DISCUSSION

The observation that the appearance of reaction centre/B875 transcripts precedes that for B800-850 is consistent with the proposal that the synthesis of the photosynthetic apparatus starts with the concomitant synthesis of reaction centres and B875 (Niederman et al., 1976; Hunter et al., 1982; Hunter et al., 1985). Results from excitation-annihilation studies on this induction system show that, at the earliest measurable stage in photosynthetic membrane assembly, energy-transfer domains are composed of 100-200 bacteriochlorophyll molecules (mainly B875 with 4-5 reaction centres (Hunter et al., 1985). More recently, it has been proposed that such domains form the building blocks for the mature photosynthetic apparatus, (Vos et al., 1986). During the next stage of photosynthetic membrane assembly, B800-850 antenna is added to existing B875 reaction-centre 'cores', and probably forms aggregates containing a minimum of 40-50 bacteriochlorophyll molecules (van Grondelle et al., 1983; Vos et al., 1986). This sequential mechanism for the assembly of photosynthetic unit has also been established for Rb.capsulatus

(Schumacher and Drews, 1978); hybridization analyses have demonstrated that a downward shift in oxygen tension in the growth medium results in increased levels of transcripts coding for reaction-centre and B875 complexes (Clark et al., 1984; Klug et al., 1985; Zhu and Hearst, 1986) and the B800-850 complex (Klug et al., 1985; Zhu and Hearst, 1986). In agreement with the work of Klug et al. (1985) we find a small lag in the rise of B800-850-specific mRNA in comparison with that for reaction centres and B875. This lag in mRNA levels occurs approximately 3h into experiments where the induction of bacteriochlorophyll occurs over a period of 19h (Ashby et al., 1987b). Here, a similar eventual level of cellular bacteriochlorophyll was achieved in 6h, which shortened the lag considerably. However, these observations are consistent with respect to cellular bacteriochlorophyll, since in each case mRNA levels encoding B800-850 apoproteins rise rapidly as bacteriochlorophyll increases to (6-8)x10^5 molecules/cell. There is clearly a close regulatory relationship between the biosynthesis of pigments and proteins of the photosynthetic apparatus. In this regard, it has been shown that mRNA specific for genes controlling bacteriochlorophyll synthesis achieves a maximum level after 90 minutes of pigment induction (Hunter and Coomber, 1988), a rise which parallels that seen for reaction centre- and B875-specific mRNA. Whether or not the lag in the rise of B800-850-specific mRNA with respect to reaction centres and B875 is indicative of different modes of control on puc and puf genes is not clear; hybridization studies such as these do not permit distinction between increased transcription of the genes or differential stability of mRNA species.

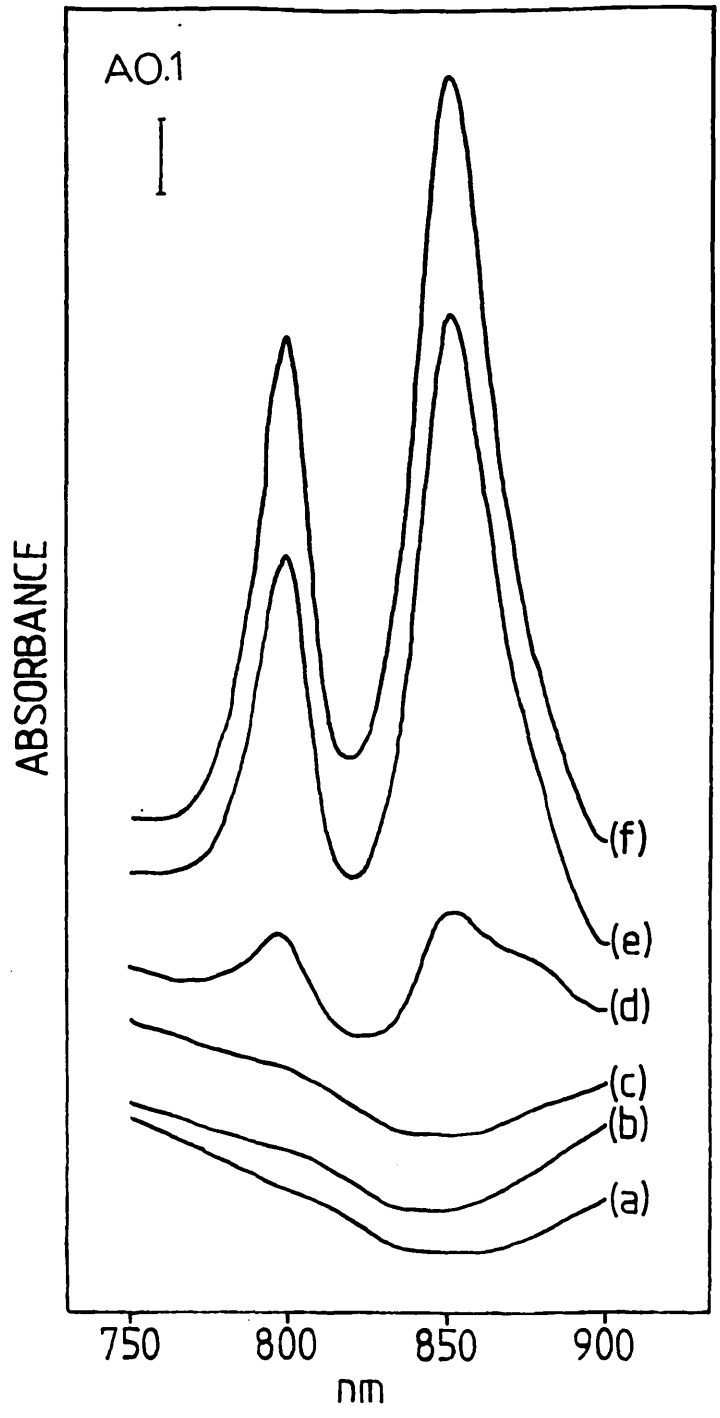


FIGURE 4.1.

Absorbance spectra of whole cells showing induction of the photosynthetic apparatus in Rb.sphaeroides

(a)-(f), Samples were removed from the culture after 0, 0.5, 1, 2, 4, and 6 h of induction respectively. The same wet wt. of cells was used for each time point.

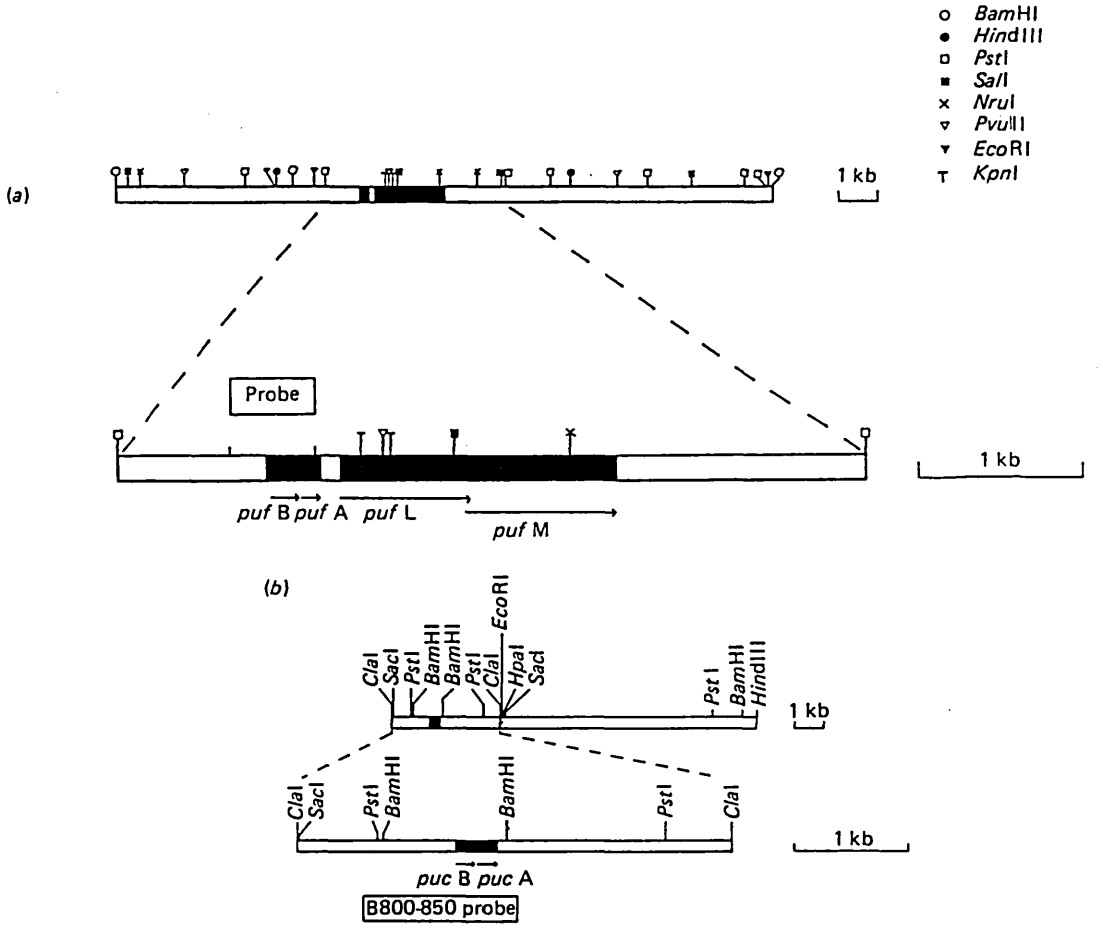


FIGURE 4.2.

Restriction maps of the cloned inserts of (a) pJW1 and (b) pMA81 showing probes used in subsequent hybridization studies and location of puf and puc genes.

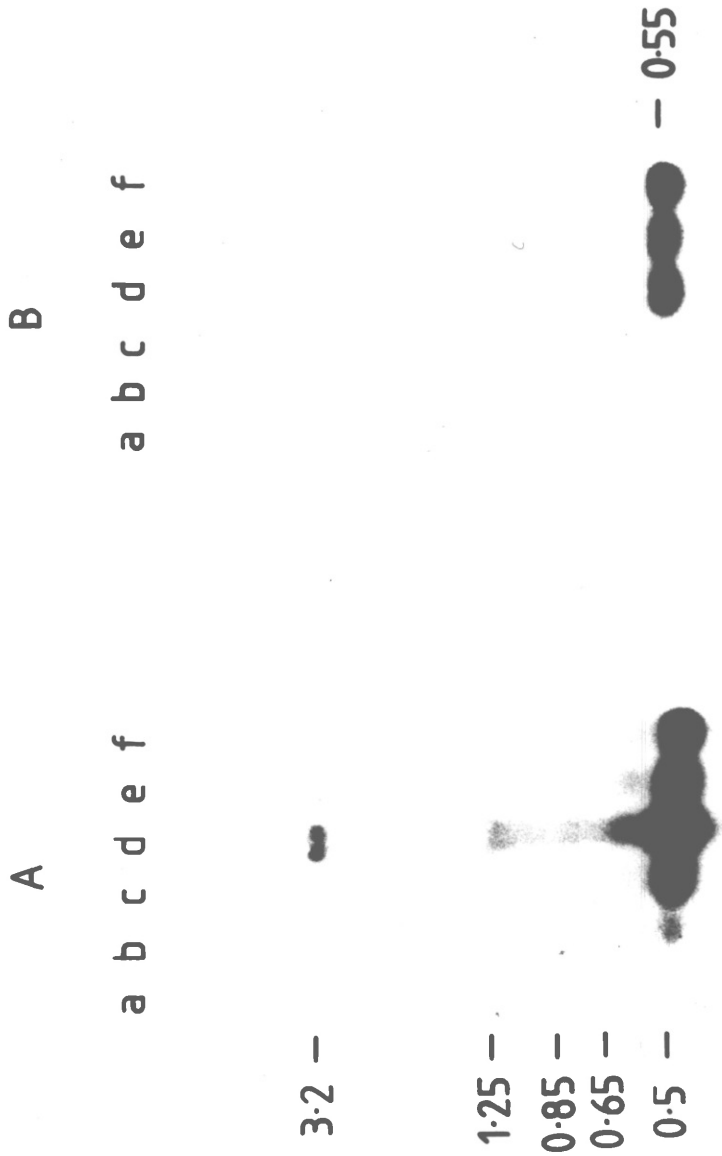


FIGURE 4.3.

Northern blots of Rb.sphaeroides RNA following pigment induction. Tracks 1 to 6, 0, 0.5, 1, 2, 4, and 6 h respectively. (a) AluI puf probe. (b) BamH1 puc probe. Molecular masses are indicated alongside the bands in kb. 5µg of RNA was loaded per well

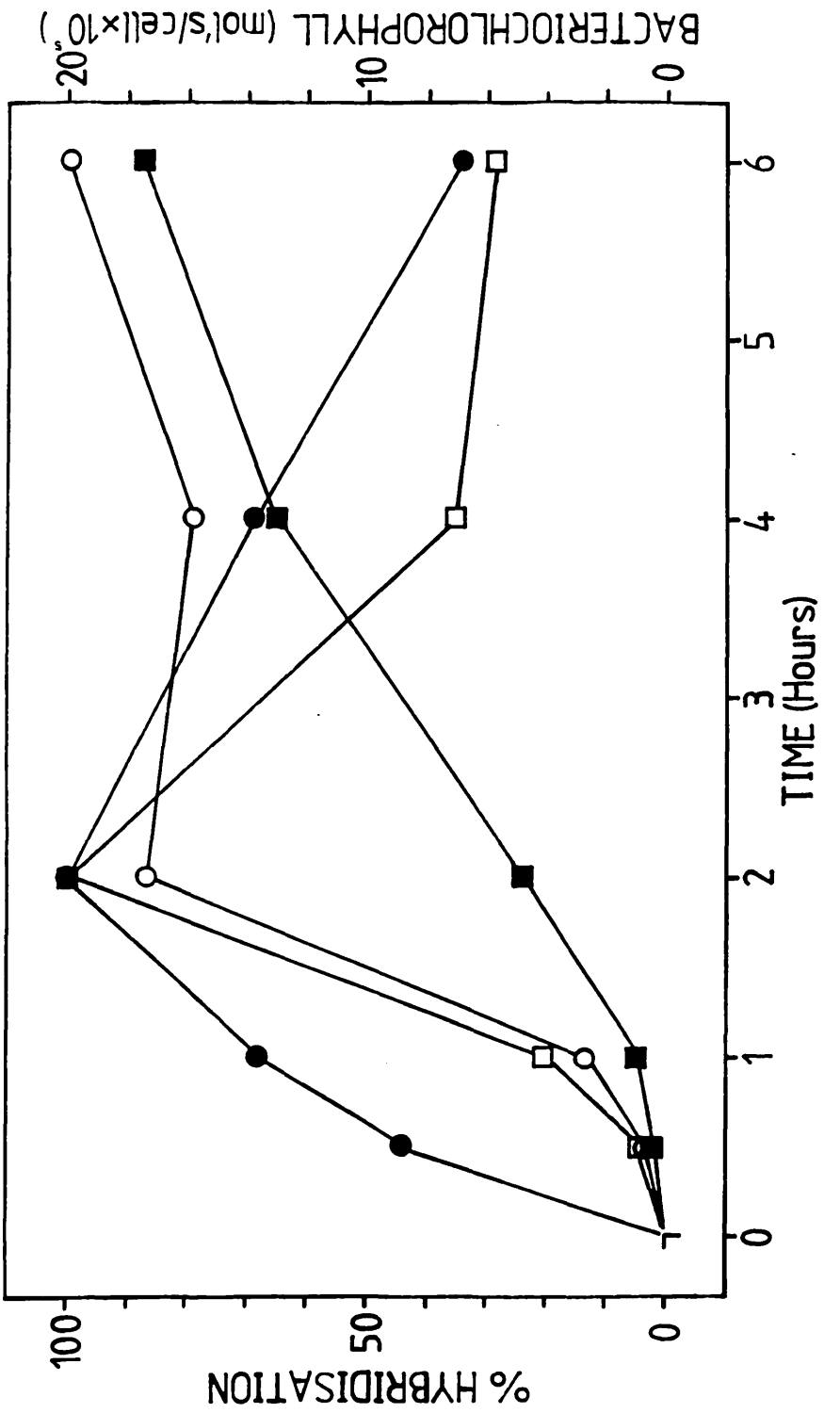


FIGURE 4.4.

Graph showing the change in levels of bacteriochlorophyll and mRNA for photosynthetic complexes.

●, 2.7kb transcript (reaction centre/B875); □ ,0.5kb transcript (B875); ○, 0.55kb transcript (B800-850); ■ , bacteriochlorophyll.

CHAPTER 5

CHARACTERISATION AND COMPLEMENTATION OF A MUTANT OF *RHODOBACTER SPHAEROIDES* WITH A CHROMOSOMAL DELETION OF THE LH2 GENES

5.1. INTRODUCTION

The photosynthetic bacterium *Rhodobacter sphaeroides* has a relatively simple photosystem consisting of the reaction centre and LH1 antenna complexes surrounded by LH2 antenna complexes (Vos *et al.*, 1986). The light harvesting antenna complexes which contain bchl are interconnected to form a highly efficient domain for light harvesting and energy transfer, the excitation energy eventually being passed to the special pair bchl of the reaction centre, where the primary photochemical event occurs. The genes encoding the reaction centre L and M, and LH1 α and β polypeptides are encoded on the *puf* operon, and the nucleotide sequences of these genes are known (Williams *et al.*, 1983; Williams *et al.*, 1984; Kiley *et al.*, 1987). The reaction centre H gene is encoded on a separate operon, *puh* (Donohue *et al.*, 1986a). The LH2 α and β polypeptides are encoded in the *puc* operon and the nucleotide sequences of these genes have been determined (Ashby *et al.*, 1987; Kiley and Kaplan, 1987).

The use of mutants for the study of excitation energy transfer has so far proved valuable (Meinhardt *et al.*, 1985; Hunter and van Grondelle 1988; van Dorssen *et al.*, 1988; Vos *et al.*, 1988) enabling a more detailed analysis of LH1, LH2, LH1/LH2 and LH1 reaction centre complexes *in situ*. The LH2 complex is possibly organised as 3 α and 3 β polypeptides (Zuber, 1986) with 6 850nm bchl's, 3 800nm bchl's and 6 carotenoid molecules: the LH2 complex constitutes the major light harvesting pigment protein complex in the membrane. This well characterised complex (Kramer *et al.*, 1984b) would therefore be an ideal model system for more detailed site directed mutational analysis of the structure and function of the LH2 antenna, particularly with regard to the importance of residues conserved in antenna polypeptides of several purple photosynthetic bacteria (Brunisholz and

Zuber,1988). In order to do this, it is necessary to construct a chromosomal deletion insertion mutant (Niaudet et al., 1982) to provide a null genetic background to act as a recipient for transferred genes. The LH2 deficient strain M21 (Ashby et al., 1987; chapter 3) has been extensively used to study the organisation of LH1/reaction centre core complexes in the photosynthetic membrane (van Dorssen et al., 1988; Vos et al., 1988), but the reversion rate (about 10^{-6}) and the unknown location of the mutation has provided an additional reason for constructing a stable, defined $LH2^-$ mutant.

In this work I describe the construction of an $LH2^-$ chromosomal deletion mutant DBC1. Deletion of the genes from the chromosome was confirmed by Southern blotting, spectral analysis and SDS-PAGE, indicating complete loss of LH2. DBC1 has been restored to wild type phenotype by the transfer of cloned genes, demonstrating that this mutant would be a suitable background for mutational analysis of the regulatory sequences or of structural genes for LH2 synthesis.

5.2. RESULTS

The 3.8kb BscI fragment containing the puc operon was subcloned from pMA81 (figure 2, chapter 3) into pAT153, a better characterised and more flexible cloning vector. Deletion of the puc operon from pMAT81 (see figure 1) was accomplished by removing a 950bp BamH1 Km^r cartridge derived from Tn903. The 4.0kb Bsc1 fragment containing the Km^r cartridge was cloned into the mobilisable suicide vector pSUP202 (Simon et al., 1983). This construct (pMSK11) was transferred into Rb.sphaeroides by conjugative transfer from E.coli strain 17-1. Photosynthetic Km^r resistant Rb.sphaeroides colonies were obtained about half of which were slightly smaller than the rest, which may be expected since $LH2^-$ strains would have a decreased light harvesting capacity, with only 330 bchl's per photosynthetic domain, instead of about 3,000 (Vos et al., 1988).

Potential deletion/insertion mutants, obtained by homologous recombination between the Rb.sphaeroides DNA flanking the Km^r

cartridge and DNA flanking the operon in the chromosome, were further identified by absence of the characteristic LH2 800nm and 850nm peaks in absorbance spectra and by probing lysed colony filters with the 950bp BamH1 puc fragment and pSUP202. Two Km^r resistant Rb.sphaeroides strains were obtained which were $LH2^-$, and showed no hybridisation to the 950bp BamH1 puc gene probe or to the pSUP202 vector probe. One of these was designated DBC1.

Figure 2 shows Southern blot analysis of Rb.sphaeroides strain DBC1 indicating that this mutant has been formed by a double crossover event replacing the 950bp BamH1 fragment containing the puc operon with a 1.25kb BamH1 fragment containing a Km^r gene. Figure 2.1. shows that the 950bp BamH1 fragment containing the puc operon which is present in the wild type chromosome, is absent from DBC1. The Southern blot probed with the 1.25kb BamH1 fragment containing the Km^r gene (figure 2.2.) shows that DBC1 contains this fragment integrated into the chromosome, and is absent from the wild type genome. The puc operon is encoded on a 15kb BscI fragment as indicated on the Southern blot probed with the 3.8kb BscI fragment from pMAT81 (figure 2.3). Note that the two BscI sites of pMAT81 (figure 2) have both fortuitously arisen from cloning partial TaqI genomic digests into the BscI site of pNH_2 (Hunter and Turner, 1988). This 3.8kb BscI fragment hybridises to a 10.5kb and 4.5kb BscI fragment from DBC1 indicating that the 15kb BscI fragment encoding the puc operon has been interrupted by the BscI site in the Km^r gene of Tn903. Figure 2.4 shows no hybridisation between DBC1 genomic DNA and the plasmid indicating that this plasmid did not integrate into the chromosome, and that the deletion of the puc operon has occured by a double recombination event replacing the 950bp BamH1 fragment with the 1.25kb BamH1 fragment containing a Km^r gene.

Absorbance spectra of wild type and DCB1 are indicated in figure 3. The wild type shows the characteristic peaks at 800nm and 850nm due to LH2 antenna complexes, as well as a shoulder at 875nm due to the LH1 antenna. DBC1 lacks the LH2 absorbance peaks of 800nm and 850nm, and exhibits an absorbance peak at 875nm which arises from the LH1 antenna, together with a small

peak near 800nm due to reaction centres. Thus the absorbance spectra indicates that the LH2 complex is not present in the membrane of DBC1 and that the LH1/reaction centre core (Vos et al., 1988) remains.

SDS-PAGE analysis of photosynthetic membranes is shown in figure 4. Track 1 shows the three low molecular weight bands that are observed in the wild type; they are from LH1 α , LH2 α and LH1 β and LH2 β superimposed, in decreasing molecular size (Broglie et al., 1980). The B800-850 α and β polypeptide were undetectable in membranes prepared from DBC1 (track 2) indicating that these polypeptides are absent because the puc gene has been lost. The remaining bands are LH1 α and LH1 β .

The puc A and B genes were transferred to DBC1 in an attempt to restore LH2 to the mutant. The plasmid pMA81 (chapter 3) which contains the puc operon on a 3.8kb BscI fragment was transferred to DBC1 by conjugal transfer from E.coli Sm10. Streptomycin resistant photosynthetic colonies were obtained. Seven colonies were cultured photosynthetically for spectral analysis. One transconjugant strain was found, DBC181, which exhibited the characteristic LH2 absorbance peaks (see figure 3). SDS-PAGE analysis of membranes from DBC181 shows that the B800-850 α and β polypeptides have been restored (see figure 4 track 3).

5.3. DISCUSSION

In this study I have reported the construction of a chromosomal deletion/insertion mutant for genes encoding the apoproteins of the LH2 complex, showing that the mutant has lost the puc genes, the polypeptides, and the intact complex. The sequence of a 1,078 bp XhoI-PvuII fragment containing the puc operon (figure 3, chapter 3), which encompases all but about 60bp of the 950bp BamHI fragment deleted from the Rb.sphaeroides chromosome (figure 1) suggests that puc A and B are the only genes encoded on the 950bp BamHI fragment as no other open reading frames are present on the XhoI-PvuII fragment. The LH2 α and β polypeptides are encoded on a single transcript of about 0.55kb (chapter 4) which has been mapped in Rb.capsulatus to start about 120bp upstream of

puc B and stop 80bp downstream of puc A (Zucconi and Beatty, 1988), and therefore encodes no other genes.

The mutant strain DBC1 was formed by a homologous double recombination event between pMSK11 and the chromosome, whereby the Rb.sphaeroides DNA flanking the Km^r cartridge of pMSK11 recombined with DNA in the Rb.sphaeroides chromosome flanking the puc operon, permanently replacing the puc operon with the Km^r gene. Single homologous recombination events could lead to the insertion of the entire construct into the chromosome at the point of cross-over. This event was found to be more common than a double recombination event and led to colonies which were kanamycin resistant, had hybridisation signals with pSUP202 and the 950bp BamHI fragment. Some of these mutants had an $LH2^-$ phenotype, presumably depending on the point of insertion.

The Km^r gene cartridge derived from Tn903 was used rather than the Km^r gene from Tn5, as Tn5 also encodes streptomycin resistance, which could have precluded the use of streptomycin resistance markers for further genetic studies of DBC1. Youvan et al. (1985) have constructed deletion/insertion mutants for the LH2 apoprotein genes of Rb.capsulatus by a similar process. They were able to use the gene transfer agent (Yen et al., 1979) specific to Rb.capsulatus to mobilise their plasmid constructs. There is no equivalent system in Rb.sphaeroides and so the mobilisation system of Simon et al. (1983) was used which can transfer pSUP202 and derivatives to a wide variety of Gram negative bacteria. E.coli strains harbouring the tra genes integrated in the chromosome are used to mobilise pSUP202 which contains a mob gene into other Gram negative bacteria (Simon et al., 1983) pSUP202 cannot replicate in Rb.sphaeroides and 'suicides' in the cell, which in the system described here means that all Km^r colonies contain the Km^r gene integrated into the chromosome. This strategy is therefore a general strategy for constructing deletion/insertion mutants in other Gram negative bacteria.

The LH2 complex has successfully been restored to DBC1 by transfer of the *puc* operon on pMA81, indicating that DBC1 would be a suitable recipient for mutagenised LH2 genes. The *puf* operon has been deleted from *Rb.sphaeroides* (Ashby *et al.*, 1988) and both the *puf* and *puc* operon from *Rb.capsulatus* (Youvan *et al.*, 1985). Bylina and Youvan (1987) have already demonstrated the use of site directed mutagenesis as a powerful tool to probe the relationship between protein structure and function by performing saturation mutagenesis of isoleucine 229 of the *Rb.capsulatus* reaction centre L subunit. Further such studies will inevitably lead to a much better understanding of how the bacterial photosynthetic apparatus functions.

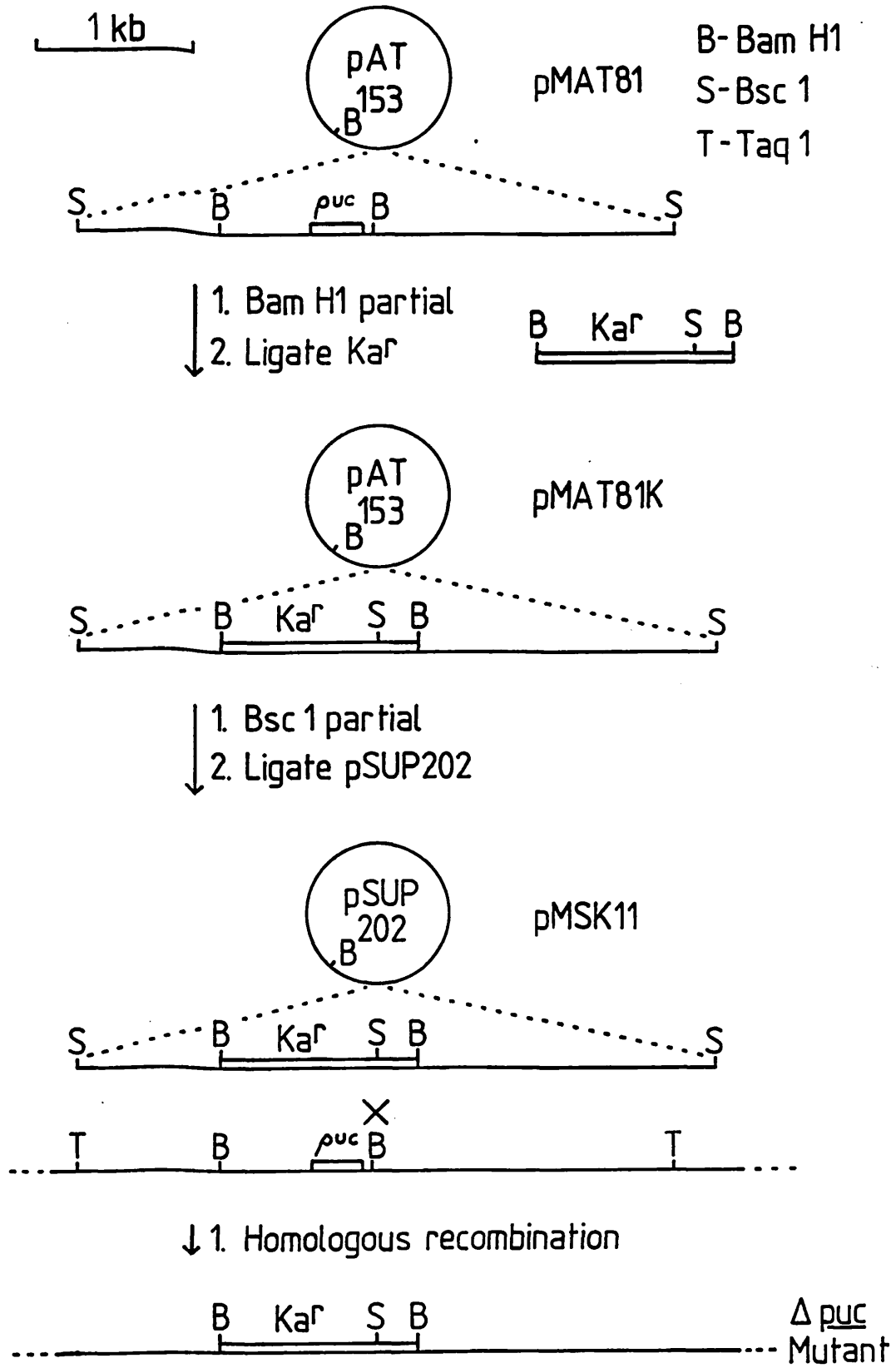


FIGURE 5.1.

Construction of DBC1, showing the restriction map of the cloned 3.8kb BscI fragment, the Km ^{R} cassette and the cassette integrated into the Rb.sphaeroides chromosome .

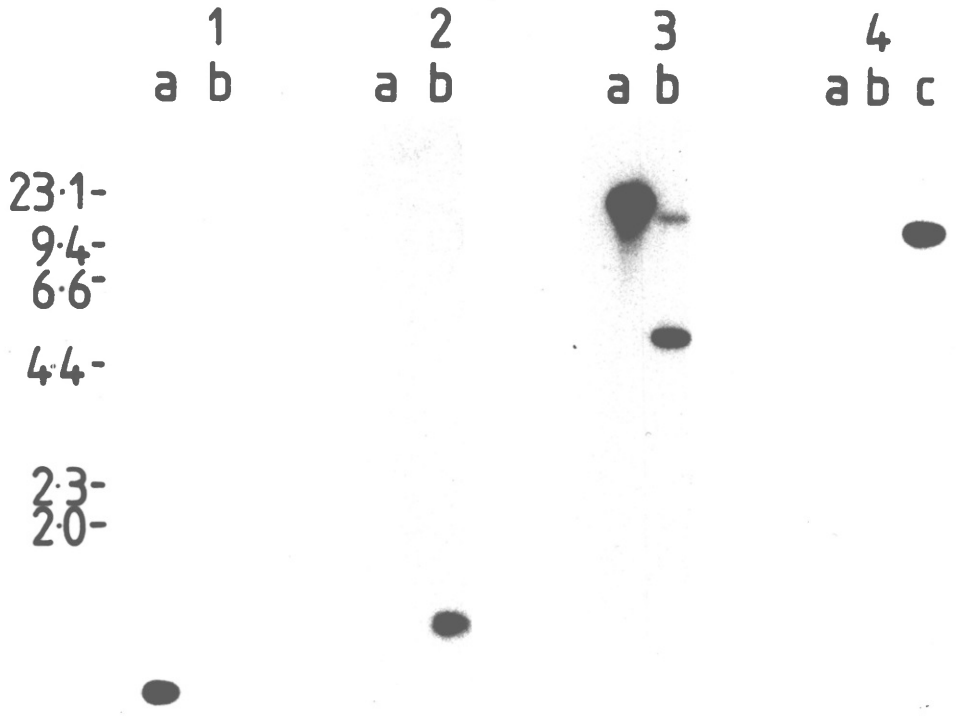


FIGURE 5.2.

Southern hybridisation of Tracks, a.) 8253 genomic DNA, b.) DBCI genomic DNA, c.) pMSK11. Blots 1 and 2 digested with BamHI, 3 and 4 with BscI Probes used. 1; BamHI 950bp puc : 2; BamHI 1.25kb Km^R : 3; 3.8kb BscI puc : 4; psup202.

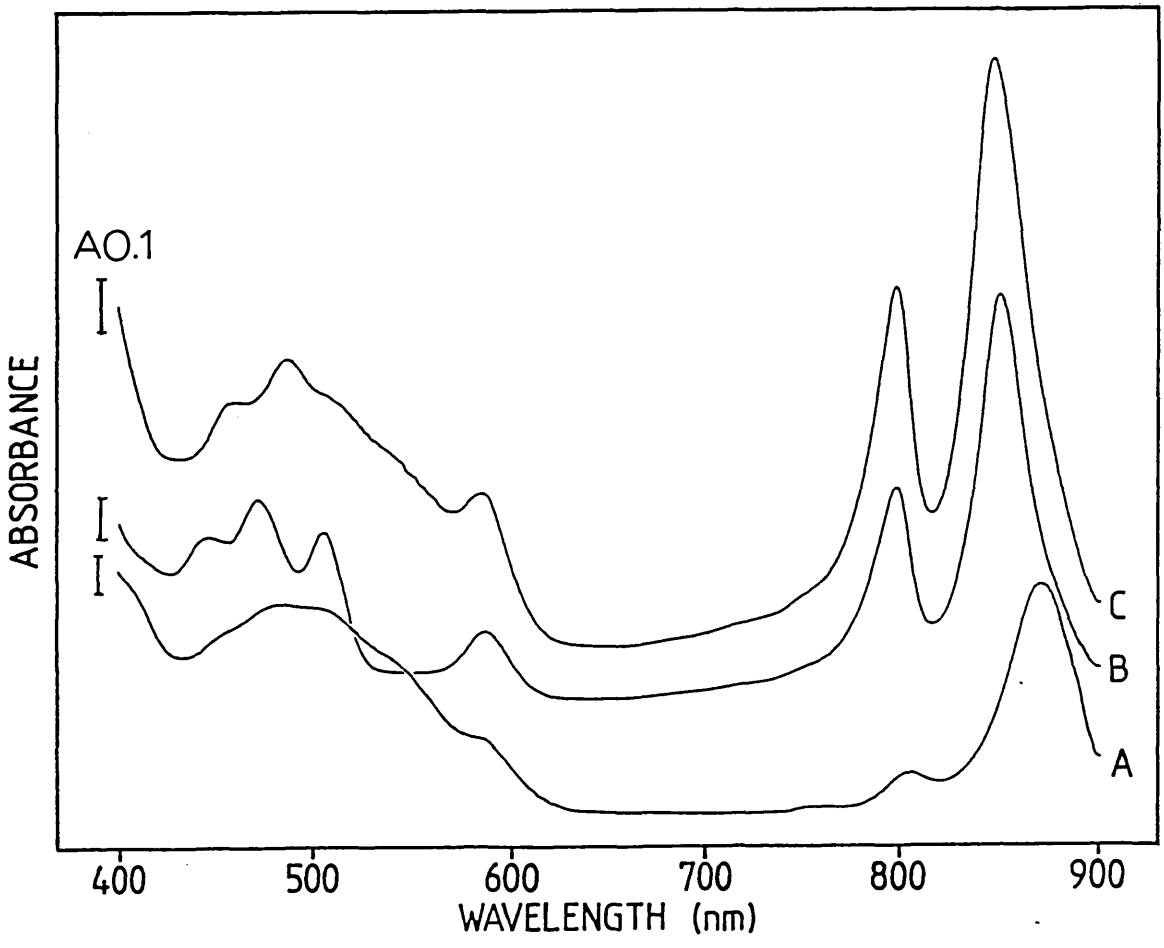


FIGURE 5.3.

Absorbance spectra of diluted membrane preparation A) DBC1 B) DBC1.81 C) 8253.

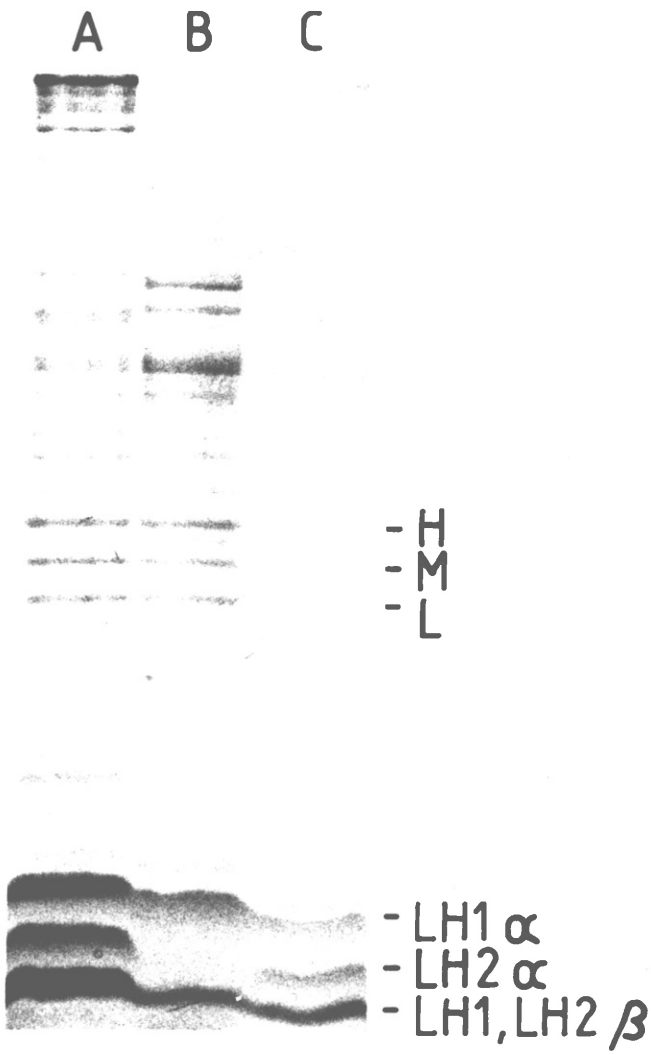


FIGURE 5.4.

SDS-PAGE of solubilised membrane. Tracks A. 8253, B. DBC1, C. DBC1.81.

CHAPTER 6

ANALYSIS OF THE PUF OPERON

6.1. INTRODUCTION

The genes for the photosynthetic apparatus have been cloned and found to be located on three distinct operons. Genes encoding reaction centre L and M and LH1 α and β polypeptides are encoded by the *puf* operon (Williams *et al.*, 1983; Williams *et al* ., 1984; Kiley *et al.*, 1987). The reaction centre H subunit is encoded by the *puh* operon (Donohue *et al.*, 1986a) and LH2 α and β polypeptides by the *puc* operon (Ashby *et al.*, 1987; Kiley and Kaplan, 1987). The *puf* and *puc* operons have also been sequenced.

Recent work has identified an open reading frame upstream of *pufB* on the *puf* operon called *pufQ* (Bauer *et al.*, 1987; Bauer *et al.*, 1988). Also, Kiley and Kaplan (1988) refer to an uncharacterised ORF upstream of *pufQ*, designated *pufR*. Little is known of the function of *pufR*, although it has been suggested as being involved in LH1 assembly (Kiley and Kaplan, 1988). The *pufQ* gene product may be involved in bacteriochlorophyll biosynthesis (Bauer *et al.*, 1988). Deletion mutants have been constructed in *Rb.capsulatus* (Youvan *et al.*, 1985) and *Rb.sphaeroides* (Davis *et al.*, 1988) which in both cases have lost all or part of the *pufQ* gene. In addition to exhibiting complete loss of the reaction centre and LH1 complexes, their mutants have greatly reduced levels of the LH2 complex, suggesting that the *pufQ* gene product must exert some trans-acting effect on either *puc* gene expression or assembly of the LH2 complex.

In *Rb.capsulatus* it has been demonstrated by constructing *lacZ* fusions that *pufQ* is transcribed as part of the *puf* operon under the control of oxygen (Bauer *et al.*, 1988). In addition, two possible promoters have been identified in the *Rb.capsulatus puf* operon, one which is oxygen regulated and one which is responsible for a low level of constitutive expression (Bauer *et al.*, 1988). As *puf* operon transcription in *Rb.capsulatus* starts 699 and 511 bp upstream of *pufB* (Bauer *et al.*, 1988), the observed 5' ends of the major 2.6kb and 0.5kb *puf* transcripts

must arise through RNA processing of the 5' end, in addition to the 3' processing of the 2.6kb transcripts which gives rise to the 0.5kb transcript. (Belasco *et al.*, 1985).

A 4.84kb *Pst*I fragment containing the *puf* operon has been deleted from the chromosome of both wild type *Rb.sphaeroides* and M21 (chapter 3) an LH2 deficient mutant (Burgess, J.G. personal communication). This fragment was replaced with a 1.25kb *Pst*I fragment encoding the Km^r gene from Tn*903*. Thus both *pufR* and *pufQ* have been removed, yielding information on the possible role of these genes, and in particular *pufR*.

In this chapter I present biochemical characterisation of these mutants and genetic analysis of the *puf* operon, concentrating on the possible functions of *pufR* and *pufQ*. Absorbance spectra of the Km^r non-photosynthetic mutants DPF1 and MDPF1 showed complete loss of any reaction centre or antenna absorption. The constituent polypeptides for these pigment-protein complexes were undetectable by SDS-PAGE, indicating that they were absent, or present at levels too low to be detected by this technique. Two open reading frames *pufR* and *pufQ* were found by sequencing the region upstream from *pufB*. Deletion of these two open reading frames from the chromosome may be responsible for the loss of the LH2 complex in DPF1 absorbance spectra. Northern blot analysis of wild type RNA, probed with 9 specific DNA fragments has identified 5 hitherto unrecognised transcripts at the 5' end of the *puf* operon, 3 of which could encode puf Q. In addition one, the 1.15kb transcript could encode *puf* R, Q, B and A. The 5' ends of these transcripts have been mapped to 690 and 748 bp upstream of *pufB*. The homology of the deduced amino acid sequences for *pufQ* and *pufR* from *Rb.sphaeroides* with *pufQ* and a putative *pufR* gene from *Rb.capsulatus* (Bauer *et al.*, 1988) is shown. Two regions of DNA in the *puf* operon have been found with homology to the *puf1* and *puf2* promoters in the *Rb.capsulatus puf* operon (Bauer *et al.*, 1988).

6.2. RESULTS

Analysis of puf operon deletion mutants:

The non-photosynthetic mutant strains DPF1 and MDPF1 were constructed by replacing the 4.48kb PstI fragment containing the puf operon with a 1.25kb PstI fragment encoding a Km^r gene from Tn903 in wild type and mutant strain M21 Rb.sphaeroides (Burgess. J.G., personal communication). The same strategy as that used to construct strain DBCI (chapter 5) was used. The deletion scheme is indicated in figure 1.

The absorbance spectra of membranes purified from wild type, DPF1 and transconjugant strains are shown in figure 2.a and spectra of M21, MDPF1 and transconjugant strains are shown in figure 2.b. Wild type Rb.sphaeroides membranes show the characteristic absorbance peaks at 800nm and 850nm due to LH2 antenna, as well as a shoulder at 875nm due to LH1. DPF1 lacks any such absorbance indicating that LH2, LH1 and reaction centres are absent. The spectra of the transconjugant strain DPF1(pSRC6) shows that the characteristic wild type spectrum has been restored to this mutant by transfer of the puf operon on pSRC6 (Burgess. J.G., personal communication).

Membranes prepared from M21 have absorbance peaks of 875nm of LH1 and a small 800nm peak due to the reaction centre. Deletion of puf genes from M21 yields MDPF1 which lacks any absorbance arising from LH1 or the reaction centre indicating that these complexes are absent. The transconjugant strain MDPF1(pSRC6) (Burgess, J.G., personal communication) has the characteristic absorbance spectrum of M21, which was restored by plasmid transfer of pSRC6.

SDS-PAGE analysis of the purified membranes is displayed in figure 3. Wild type Rb.sphaeroides (track A) exhibits bands for reaction centre H, M and L and the low molecular weight antenna polypeptides LH1 α , LH2 α , with LH1 β and LH2 β superimposed, in order of decreasing apparent molecular weight (Broglie et al., 1980). In track B all of

these polypeptides are undetectable in membranes prepared from the deletion strain DPF1, whereas membranes prepared from the transconjugant strain DPF1(pSRC6) contain all of the polypeptides from the light harvesting antenna and reaction centre complexes (track C). M21 membranes, shown in track D. contain polypeptides for LH1 and reaction centre, which are undetectable in the deletion strain MDPF1 (track E.); they are restored in transconjugant strain MDPF1(pSRC6) (track F).

In order to investigate further the lack of LH2 in deletion strain DPF1, Northern blot analysis of RNA from a culture undergoing oxygen regulated pigment induction (Niederman et al., 1976) was undertaken (Coomber, S.A., personal communication). The blot was probed with a 950bp BamH1 fragment containing the puc genes (chapter 3), and it was apparent that the 0.5kb puc transcript was induced in DPF1 when the degree of aeration of the culture is greatly reduced, as previously shown for wild type Rb.sphaeroides (chapter 4).

DNA Sequencing Upstream of puf L.

The deletion of DNA upstream of pufB clearly disrupts some element involved in the assembly of the LH2 complex, so the sequence of a 2,228 BamHI-KpnI fragment was determined in order to identify any open reading frames.

The map of a 2,228 bp BamHI-KpnI restriction fragment subcloned from pJW1 (Williams et al., 1983) is shown in figure 4 which displays the fragments subcloned into M13 mp18 and mp19 for sequence analysis. The sequence of the BamHI-KpnI fragment is shown in figure 5. Open reading frames pufR, pufQ, which overlap by 4 nucleotides, and ORF K (Bauer et al., 1988; Kiley and Kaplan., 1988) are indicated with possible Shine-Dalgarno ribosome binding sites. These open reading frames code for polypeptides of 110, 77 and 20 amino acids respectively. The position of pufR and pufQ relative to pufB is shown in figure 7.

Analysis of puf RNA.

Northern analysis of RNA prepared from Rb.sphaeroides cells after 2 hours of pigment induction is shown in figures 6A and 6B. RNA was prepared from Rb.sphaeroides 2 hours after pigment induction because the level of puf encoded mRNA is highest at this time (chapter 4). The DNA fragments from the BamH1-Kpn1 fragment used as probes are indicated in figure 4. 6 bands corresponding to at least seven RNA species are apparent, and of the following sizes; 2.6kb, 1.15kb, 0.87kb, 0.61kb, two at 0.48kb., and 0.35kb. The bands at 0.87kb and 0.61kb are very faint. The 2.6kb and intense 0.48kb transcripts are the previously characterised puf operon transcripts encoding puf B, A, L, M and X and puf B and A respectively (Zhu et al., 1986b) with 5' ends mapping 104 and 75bp upstream of pufB respectively (Zhu et al., 1986b). The 3' end of the 0.35kb fragment mapped upstream of the HincII site, and the minor 0.48kb transcript maps past the fifth BstN1 site. The end of the 0.87kb transcript was not mapped. The start of the 0.61kb band mapped downstream of the fifth BstN1 site. The transcripts are indicated in figure 8. The crude mapping using DNA fragments to probe northerns has several limitations. For example transcripts extending a few bp past the restriction site may appear to give a negative result, leading to the conclusion that the 5' or 3' end does not extend after this site. For this reason mung bean nuclease analysis was attempted.

The 5' ends of the 1.15kb, 0.87kb, minor 0.48kb and 0.35kb transcripts were mapped by mung bean nuclease analysis using M13 clones of the BamHI-PstI fragment (figure 7) and a 160bp PstI-Sau3A fragment was also used in preliminary analysis (data not shown). The 5' end of the 1.15kb transcript mapped 748bp upstream of pufB, and the 0.35kb, minor 0.48kb and 0.87kb transcripts approximately 690bp upstream of pufB. The length of the 29bp protected fragment shown in figure 9 is an estimate and could be between 35 and 15 bp. The start of these transcripts is indicated on figure 8.

The map showing the BamHI-KpnI fragment and open reading frames with the RNA transcripts has been drawn from the Northern mapping experiments and mung bean nuclease analysis, taking into account the observed sizes of the transcripts (figure 8). It is apparent that the 1.15kb transcript could encode puf R, Q, B and A. The 0.87kb transcript could encode puf Q, B and A. The 0.61kb transcript could encode puf B and A and the minor 0.48kb transcript could encode pufQ. The 0.35kb transcript does not appear to encode any complete open reading frames.

Sequence Homologies.

The homology of the puf1 and puf2 promoters in the puf operon of Rb.capsulatus (Bauer et al., 1988) with two regions of the Rb.sphaeroides puf operon at around 880 and 1,050bp downstream of the BamH1 site and 680 and 494bp upstream of pufB is shown in figure 9. Homology between the derived amino acid sequences of pufR and pufQ with a putative pufR gene and pufQ from Rb.capsulatus (Bauer et al., 1988) is shown in figure 10.

6.3. DISCUSSION

The construction of two non-photosynthetic strains of Rb.sphaeroides (DPF1 and MDPF1) from which a 4.84kb PstI fragment encoding the puf operon has been removed from the chromosome, and replaced with a Km^r gene has been reported (Burgess, J.G. personal communication). The phenotype of the parent strains was restored by conjugative transfer of pSRC6 containing the puf operon demonstrating the potential use of these strains DPF1 and MDPF1 as blank genetic recipients for cloned puf operon DNA during mutational analysis of the puf operon.

Chromosomal deletion/insertion mutations of the whole puf operon have been constructed in Rb.capsulatus (Youvan et al., 1985) and of part of the puf operon from Rb.sphaeroides (Davis et al., 1988). The mutants that they constructed were all non-photosynthetic but the deletion mutants

constructed from wild type strains possessed a small, but measurable amount of LH2 complex in the photosynthetic membrane. However DPF1 did not possess any detectable LH2 complex as judged by spectral analysis of absorbance spectra or of the constituent apoproteins by SDS-PAGE. One important difference between DPF1 and the mutants constructed by Youvan *et al.* (1985) and Davis *et al.* (1988) is the amount of DNA deleted upstream of *pufB*. The *Rb.capsulatus* (Youvan *et al.*, 1985) mutant U15 had a deletion as far as 215bp upstream of *pufB*; for the *Rb.sphaeroides* (Davis *et al.*, 1988) mutant PUFB1, DNA was deleted 282bp upstream of *pufB*. In this work the deletion removes DNA 660bp upstream of *pufB*. This leads to the conclusion that there may be an important gene between 660bp and 282bp upstream of *pufB* involved in pigment biosynthesis and/or LH2 production.

Two open reading frames upstream of *pufB* have been identified, denoted *pufR* and *pufQ*, of 110 and 77 amino acids respectively. The *pufQ* gene is encoded by 3 transcripts, one of which could also encode *pufR*. The *pufQ* gene has been identified in *Rb.capsulatus* and shown to be transcriptionally linked to the *puf* operon. Transcripts encoding the *puf* operon originate upstream from *pufQ* (Bauer *et al.*, 1988). The functions of *pufQ* and *pufR* have not been confirmed but it is clear that they are both involved in the formation of LH2 complexes. They are clearly not involved in the transcription of the *puc* operon or of bacteriochlorophyll biosynthesis genes as *puc* and *bch* transcripts are present (data not shown). Dierstein, (1983) and Klug *et al.*, (1986) have shown that pigment protein complexes are not formed in mutants unable to synthesise bacteriochlorophyll, and that the constituent light harvesting apoproteins are made but very rapidly degraded. A pigment is present in the medium which absorbs at 640nm (data not shown) presumably a precursor of bacteriochlorophyll, which leads me to believe that *pufQ* and *pufR* are involved in bacteriochlorophyll biosynthesis as has

been suggested by Bauer et al. (1988). The LH2 apoproteins would not be detectable in DPF1 if they are rapidly degraded (Dierstein, 1983; Klug et al., 1986), so in this respect DPF1 behaves as a bchI$^-$ mutant. The involvement of pufR and/or pufQ in the correct assembly or insertion of the LH2 complex into the membrane cannot be ruled out.

The homology between the derived amino acid sequence of pufR and pufQ of Rb.sphaeroides with pufQ and putative pufR genes from Rb.capsulatus (Bauer et al., 1988) indicate that the genes designated pufQ and pufR by Bauer et al., (1988) and Kiley and Kaplan (1988) have been identified. Homology was found with the puf1 and puf2 promoters of Rb.capsulatus (Bauer et al., 1988). The puf1 promoter is the oxygen regulated promoter of the puf operon of Rb.capsulatus which by homology with the Rb.sphaeroides sequence maps approximately to the 5' end of the 0.35kb, minor 0.48kb and 0.87kb transcripts 680bp upstream of pufB. This would indicate that this is where initiation of these transcripts occurs, and probably for oxygen regulated transcription of the entire puf operon. No transcripts were detected arising from the putative low level constitutive puf2 promoter, although this may be because the level of gene expression from this promoter may be insignificant during pigment induction and/or too low for detection by the methods I have used. Further analysis of transcription of the puf operon from these promoters requires the construction of fusions of lacZ to genes of the puf operon and subsequent deletion analysis to map promoters, as has been demonstrated in Rb.capsulatus (Bauer et al., 1988). No sequence homology was found upstream of the 5' end of the 1.15kb transcript encoding pufR with either of the puf1 or puf2 promoters from Rb.capsulatus (Bauer et al., 1988). The promoter for this transcript still requires identification.

If transcription of the puf operon initiates from the puf1 promoter, then there must be a complicated mechanism of RNA processing to account for the observed transcripts. The 5'

ends of the major 0.48kb and 2.6kb transcripts have been mapped (Zhu et al., 1986b) and found to be separated by 29bp which would seem to suggest differential transcription initiation. The 0.87kb transcript could arise from rapid processing of a putative transcript encoding the whole puf operon of about 3.2kb, although this latter transcript has not been observed. The 0.61kb transcript could arise from processing of the 3.2kb or 2.6kb transcripts. The 0.35kb transcript could arise from processing of the 3.2kb, 0.87kb or minor 0.48kb transcripts. The minor 0.48kb transcript could arise from processing of the 0.87kb or 3.2kb transcripts upstream of pufB. It is possible to imagine a mechanism whereby the putative 3.2kb transcript is rapidly processed upstream of pufB to the minor 0.48kb transcripts encoding pufR and Q and the 2.6kb transcript encoding puf B, A, L, M and X. The 2.6kb transcript could then be processed by 3' exoribonucleases as described by Belasco et al, (1985) to the major 0.48kb transcript encoding pufB and A, although there may be some 5' processing of the 2.6kb transcript to account for the observed 5' ends (Zhu et al., 1986b). The 0.87kb and 0.61kb transcripts may represent alternative processing pathways of the 3.2kb transcript. The 0.35kb transcript may only be a breakdown product of the minor 0.48kb transcript formed by 3' exoribonucleases.

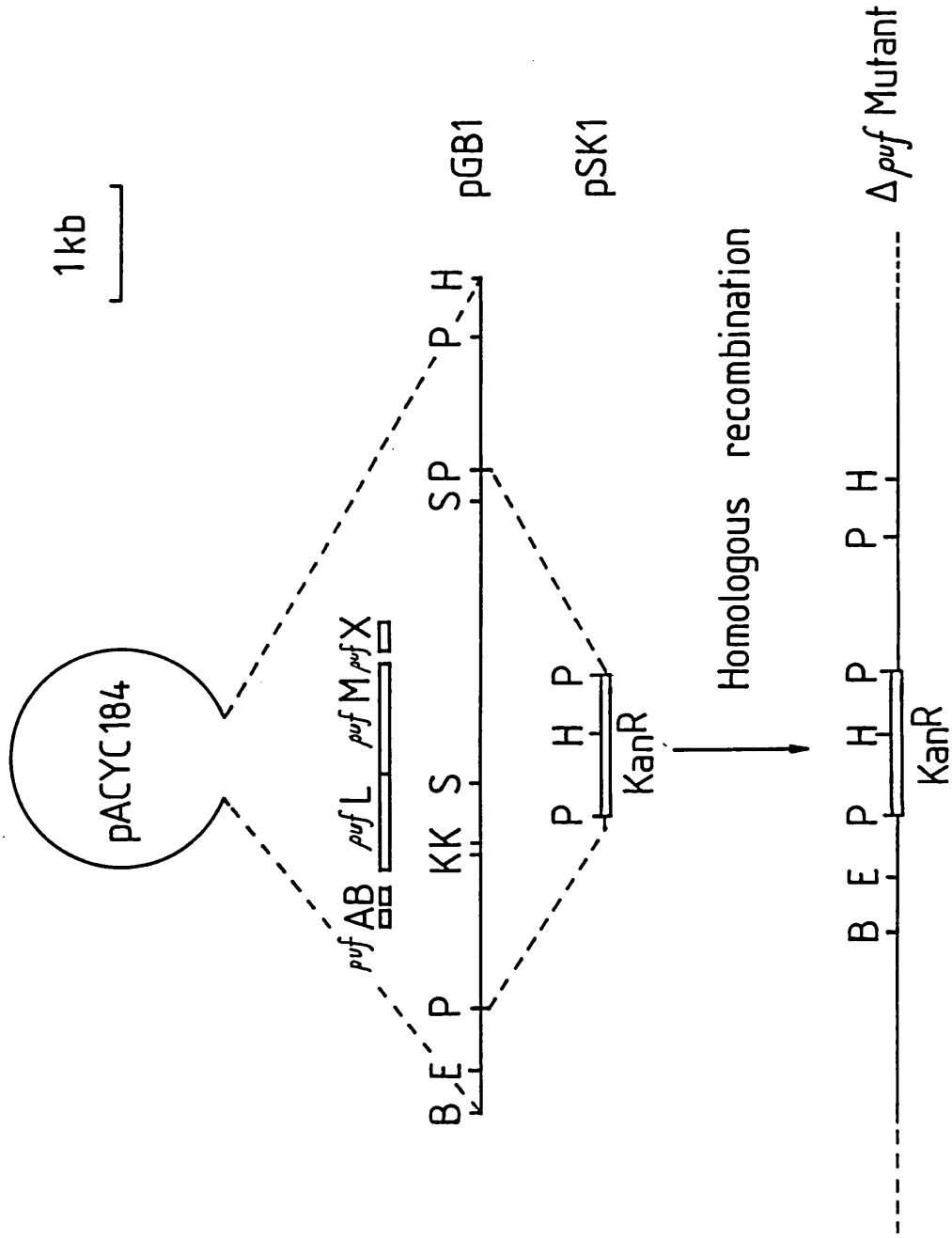


FIGURE 6.1.

Restriction map of pGB1 containing the puf operon, and of the deletion mutant DPF1. (Burgess, J.G. personal communicaton). Restriction enzyme sites: B-BamHI; E-EcoRI; H-HindIII; K-KpnI; P-PstI; S-SalI.

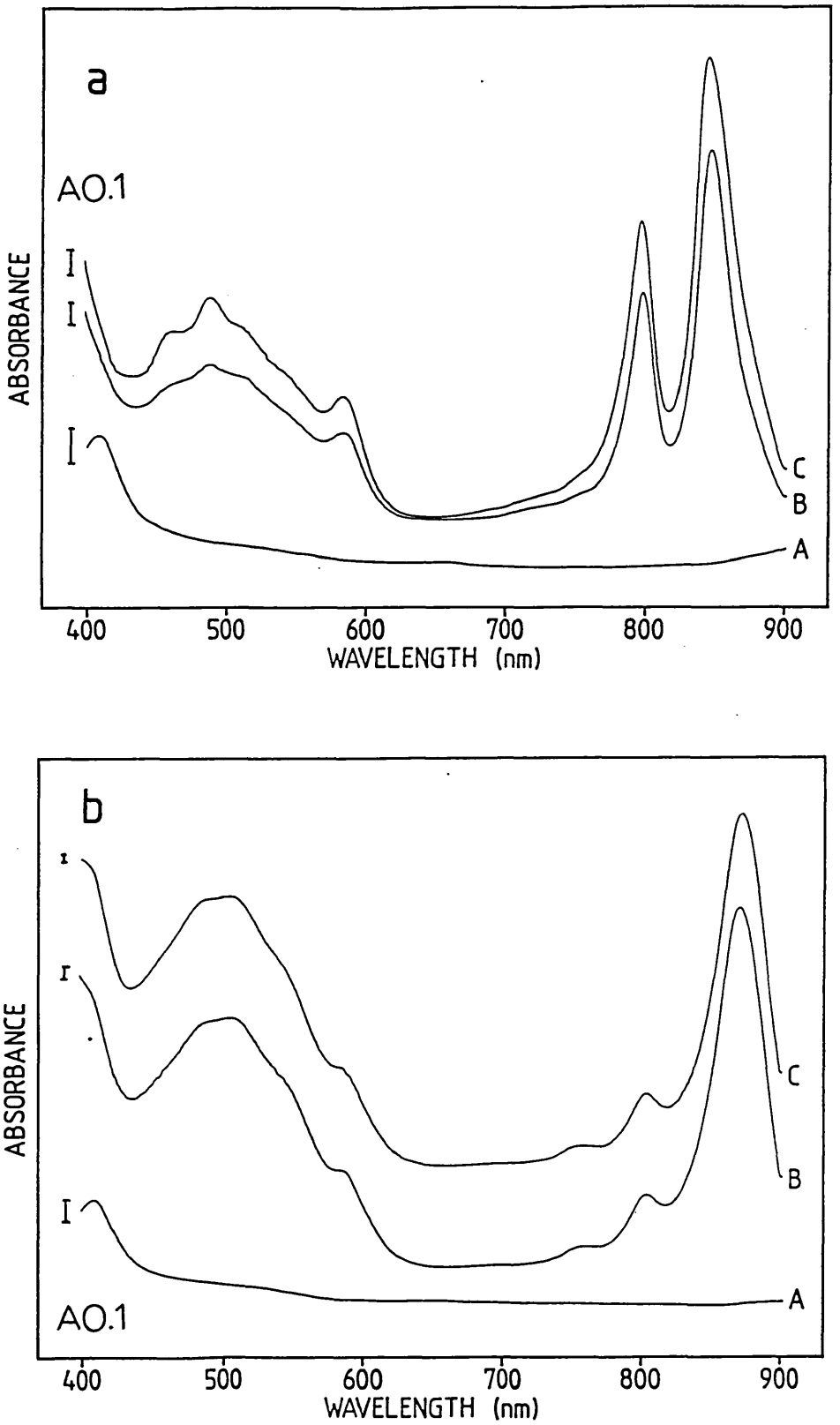


FIGURE 6.2.

Absorbance spectra of purified membranes a.) A-DPF1; B-DPF1(SRC6); C-Wild type. b.) A-MDPF1; B-MDPF1(SRC6); C-M21.

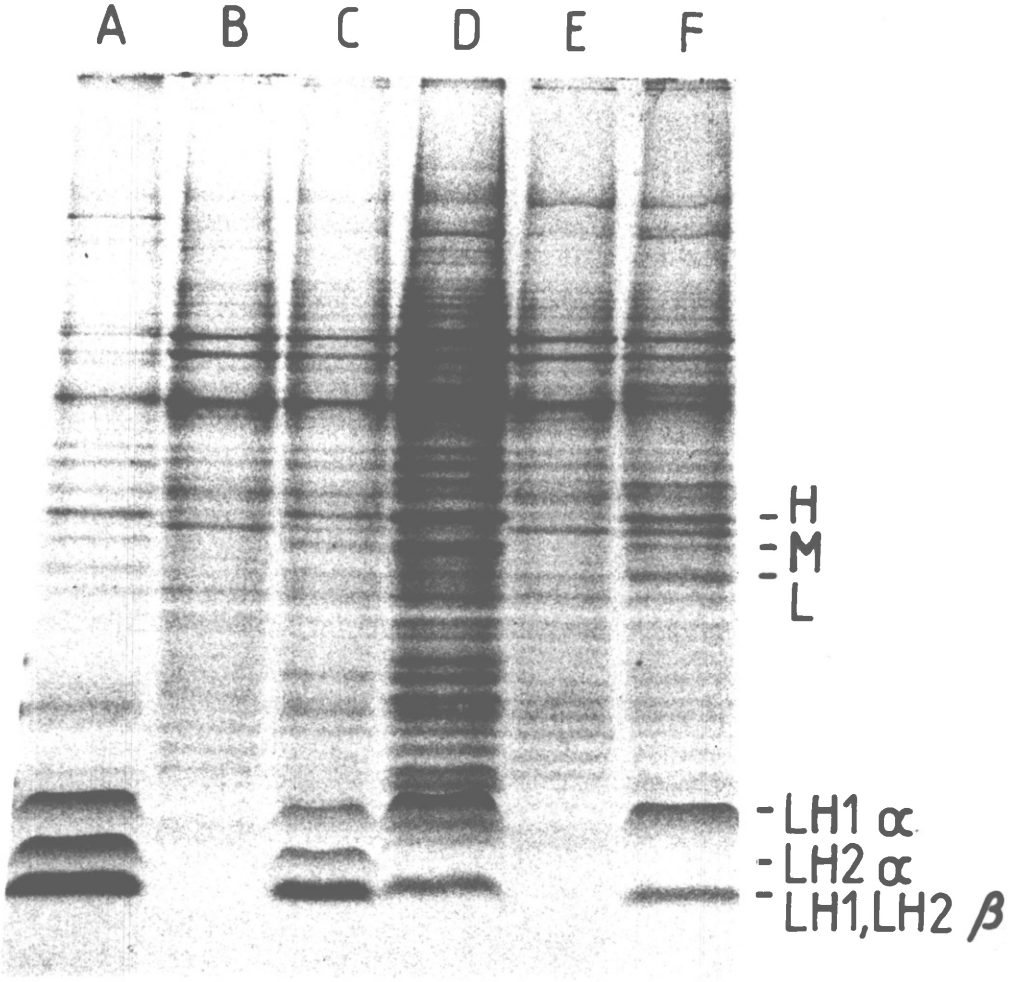


FIGURE 6.3.

SDS-PAGE of purified membranes. A-Wild type; B-DPF1; C-DPF1(SRC6); D-M21; E-MDPF1; F-MDPF1(SRC6).

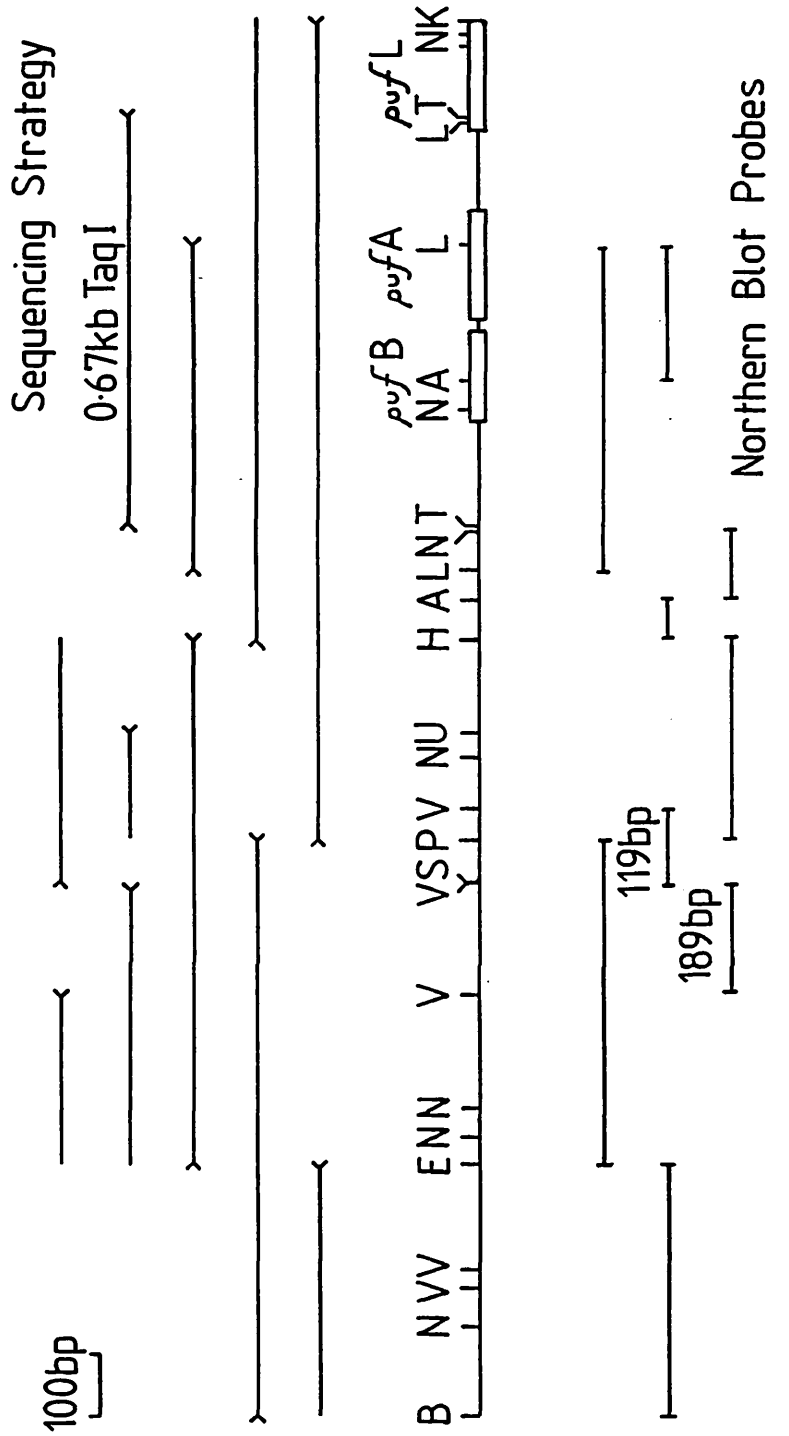


FIGURE 6.4.

Map of the 2,228bp BamHI-Kpn1 fragment containing the upstream portion of the puf operon. The fragments above the map were those used for sequencing of this fragment, the arrows indicating the direction of sequencing. The fragments below the map are those used as probes for Northern blotting. Restriction enzyme sites: A-AccI; B-BamHI; E-EcoRI; H-HincII; K-KpnI; L-AluI; N-BstNI; P-PstI; S-SmaI; T-TaqI; U-Sau3A; V-AvaI. Not all of the TaqI and Sau3A sites are indicated.

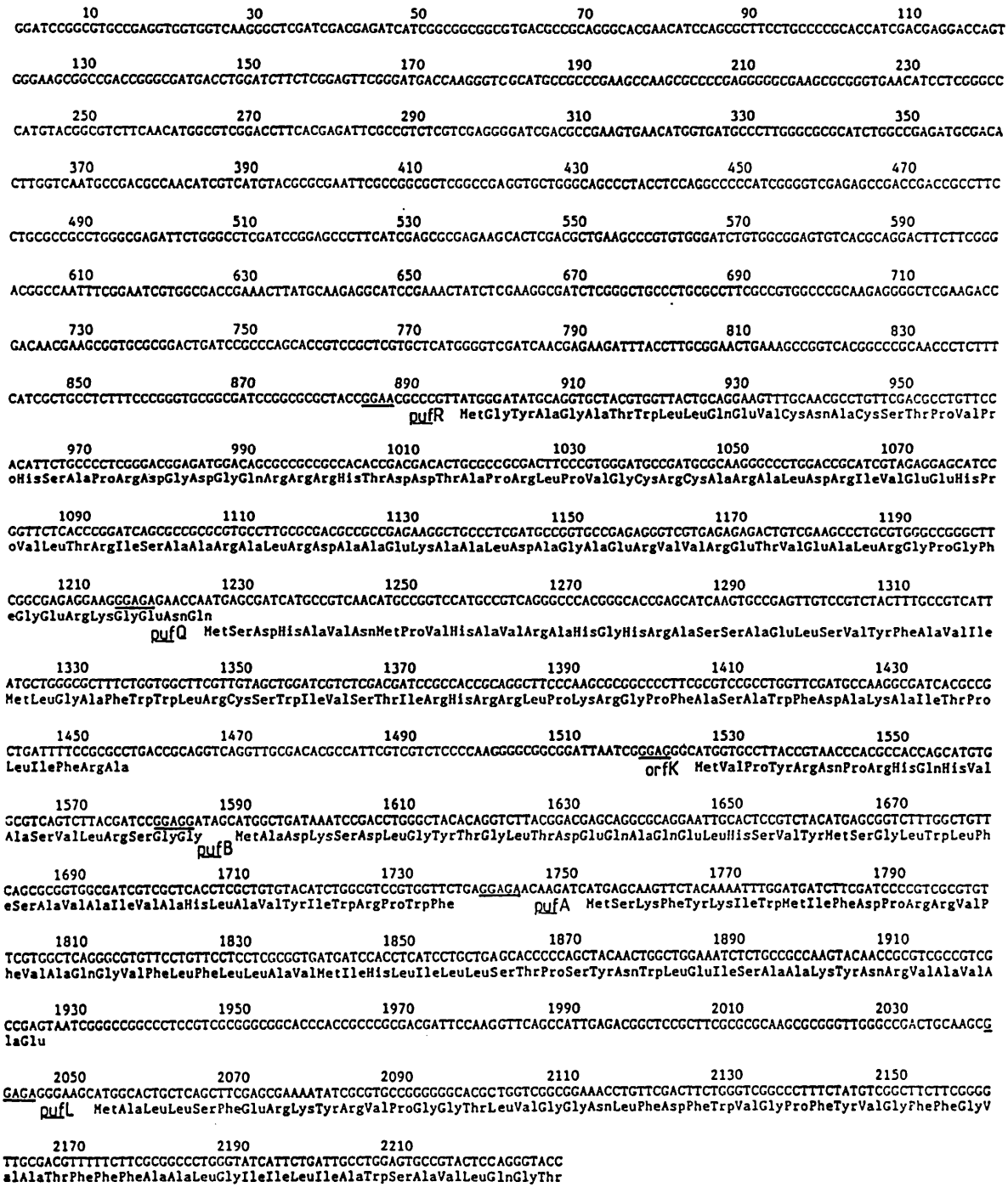


FIGURE 6.5.

Nucleotide sequence of the 2,228bp BamHI-KpnI fragment showing the deduced amino acid sequence of the puf R, puf Q, orf K, puf B and puf A genes and the upstream portion of the puf L gene. Possible ribosome binding sites are underlined.

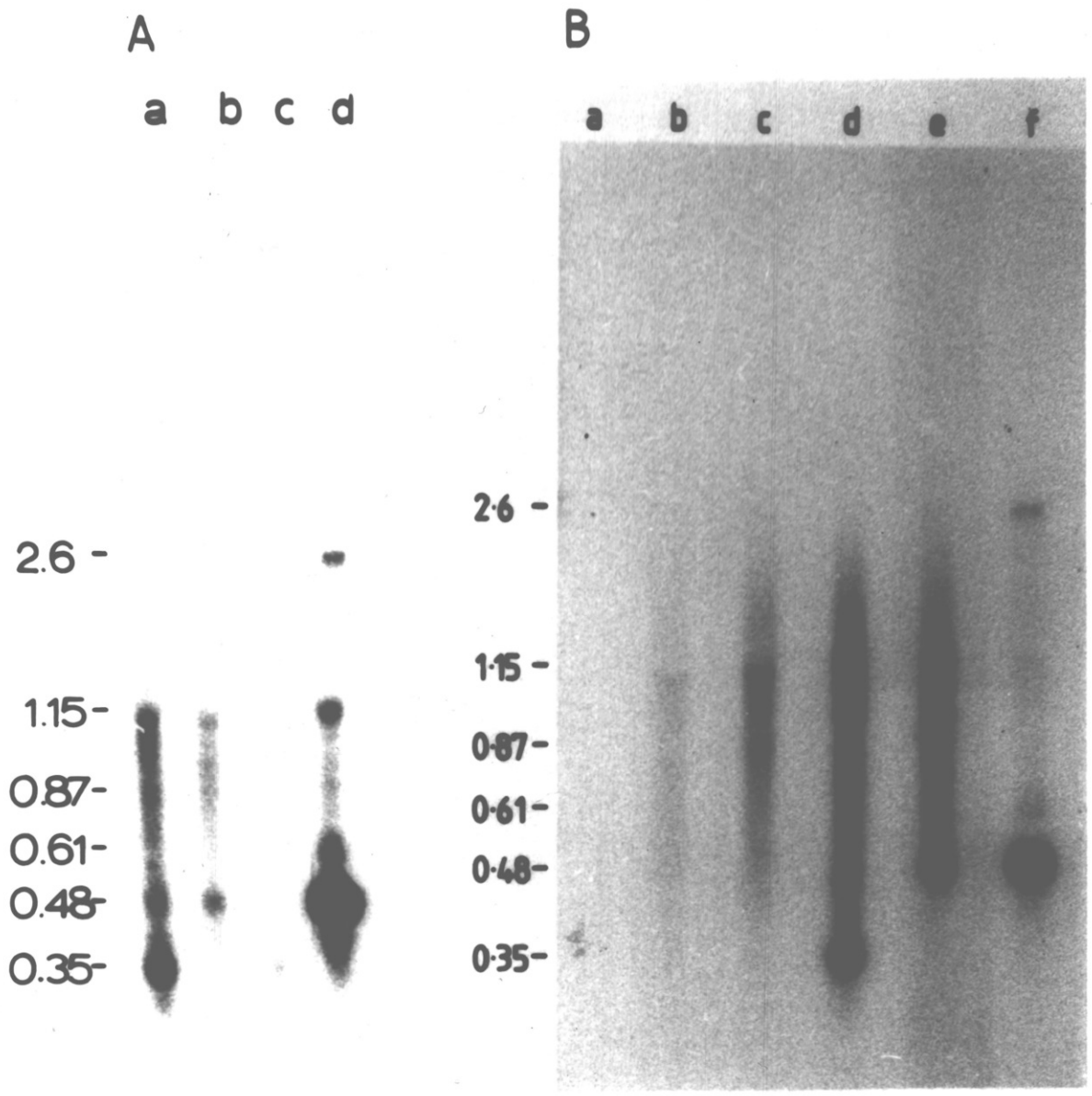


FIGURE 6.6.

Northern blot analysis of wild type Rb.sphaeroides RNA prepared 2 hours after pigment induction. DNA probes indicated on figure 4. A.) a,PstI-HincII; b,HincII-AccI; c,AvaI 119bp; d,AluI. B.)a,EcoRI-BamHI; b,EcoRI-PstI; c,AvaI 189bp; d,AvaI 119bp; e,AccI-BstNI; f,AccI-AluI. The size of the bands is shown in kilobasepairs.

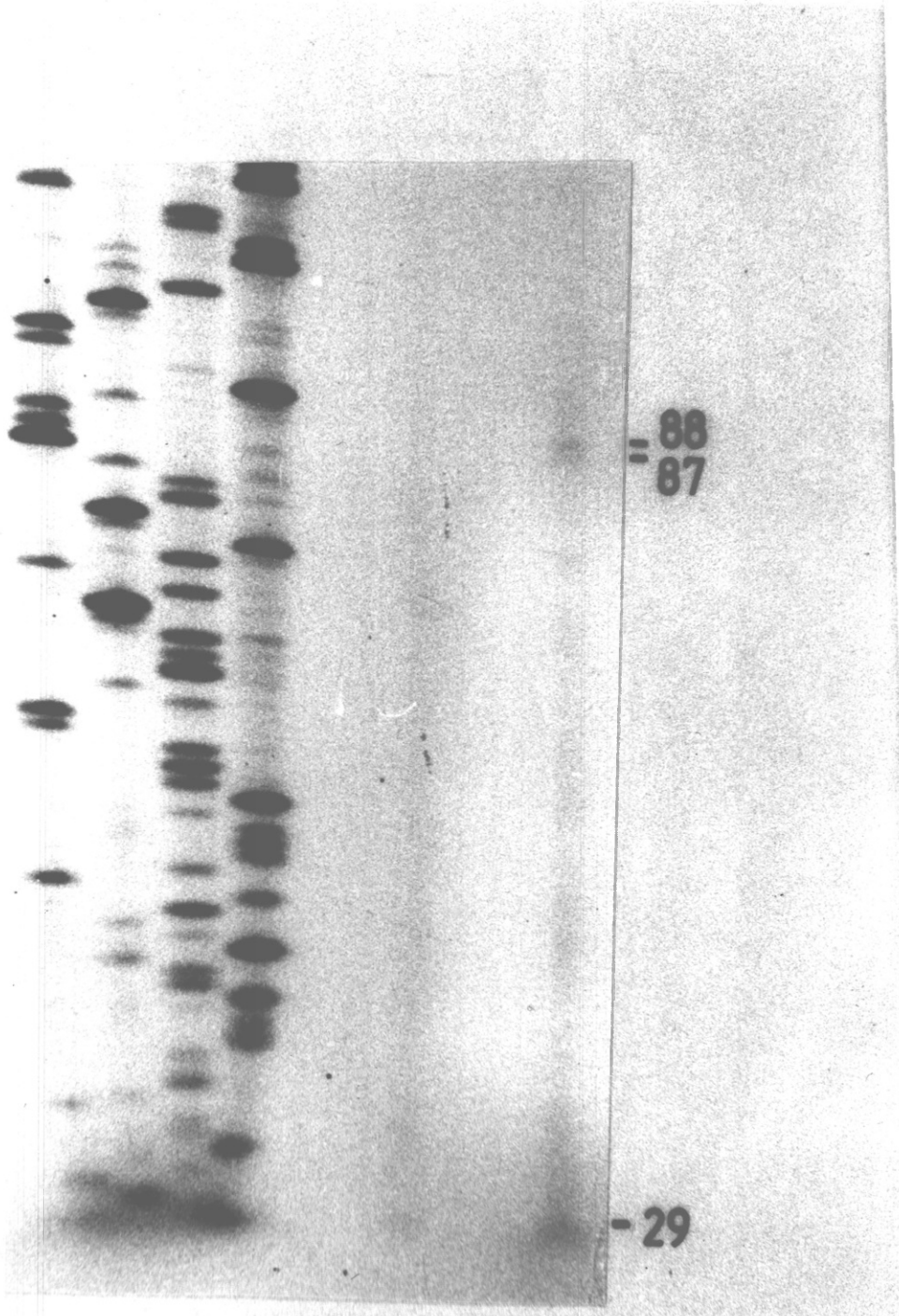


FIGURE 6.7.

Mung bean nuclease analysis of wild type *Rb.sphaeroides* RNA prepared 2 hours after pigment induction, RNA was hybridised with the 928bp BamHI-PstI fragment cloned in M13 mp18. The size of the bands is shown in basepairs.

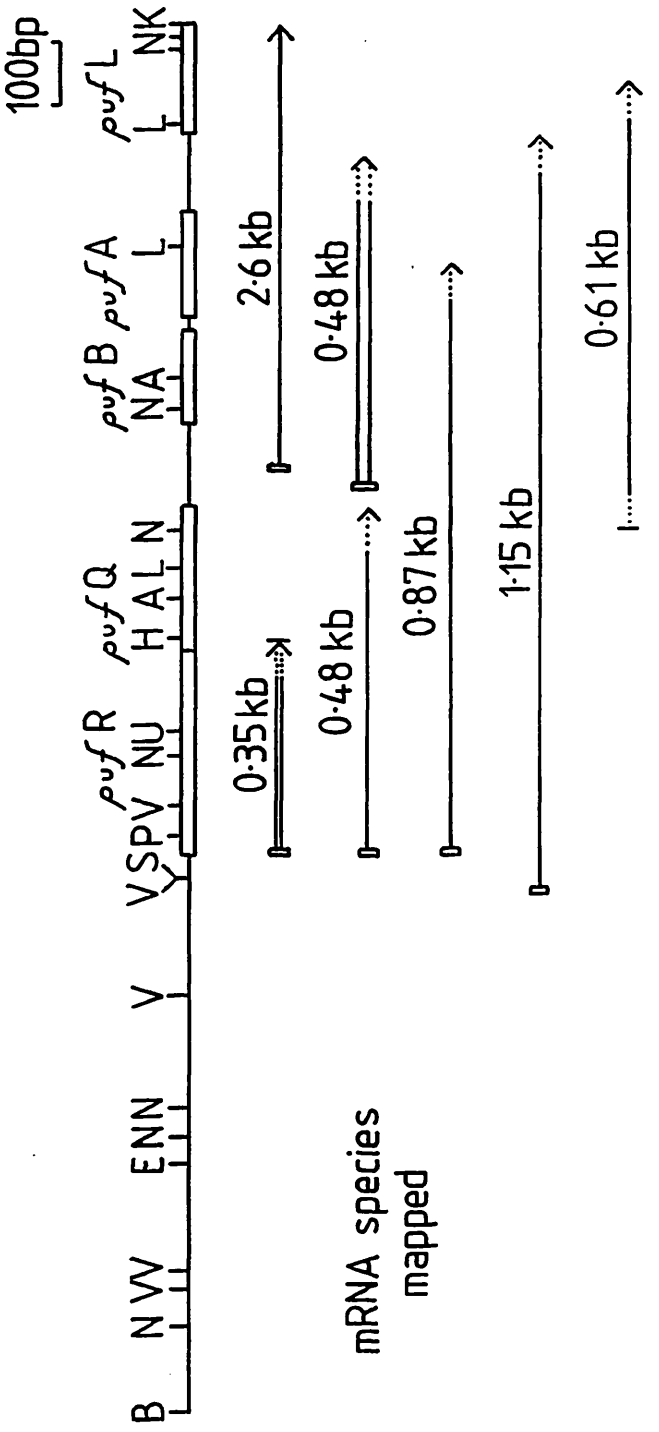


FIGURE 6.8.

Map of the 2,228bp BamHI-KpnI fragment showing the position of the puf R and puf Q genes. The position of the mRNA species mapped is shown mRNA mapping ▯-5' end mapped; |-limit of mRNA from Northern mapping; ····· approximate end from size of mRNA. Restriction enzyme sites: A-AccI; B-BamHI; E-EcoRI; H-HincII; K-KpnI; L-AluI; N-BstNI; P-PstI; S-SmaI; U-Sau3A; V-AvaI.
2.6kb and abundant 0.48kb 5'ends mapped by Zhu et al.(1986b).

```
                                                                  870                 890                 910
Rb. sphaeroides                                    CCCGGGTGCGGCGATCCGGCGCGCTACCGGAACGCCCGTTATGGGATATGCAGG
                                                   *** ** ********** ***** ** ** *** * ***** **    **
Rb. capsulatus  puf 1  CCCCGGCGCGGCGATCCGCCGCGACGGGCACCCCCTTCATGGGTTACATGGG
                        -50        -40        -30        -20        -10        +1

                                                                  1050                1070                1090
Rb. sphaeroides                                    TGCGCAA-GGGCCCTGGACCGCATCGTAGAGGAGCATCCGGTTCTCACCCGGA
                                                   ******  *** ********** *****   * * ** *********** **** ****
Rb. capsulatus  puf 2  TGCGCAAGGCGGCTCTGGACCGGATCGTGTCGCAACACCCGGTTCTGACACGGA
                        -50        -40        -30        -20        -10        +1
```

FIGURE 6.9.

Homology between the puf$_1$ and puf$_2$ promoters of the Rb.capsulatus (Bauer et al., 1988) and Rb.sphaeroides DNA. The number above the sequence indicates the position of the Rb.sphaeroides DNA on the sequence in figure 5. The numbers below the sequence indicate the start of the 5' ends of the transcripts in Rb.capsulatus (Bauer et al., 1988).

puf R *Rb. sphaeroides : Rb. capsulatus*

```
M G Y A G A T W L L Q E V C N A C S T P V P H S A P A D G D
      *     |*****|**|       |  |   |     *       | |
    M G S V Y L L Q E I C N G L F D A L F N I L P L A S E

G Q R R R H T - D D T A P R L P V G C R C A R A L D R I V E
  |         *   | |   *       |   |             *****|*|
M D S A A A T P G N L A S X H A W D A D A E A A L D R I V S

E H P V L T R I S A A R A L R D A A E L A A L D A G A E R V
| ********** |  ***********  ********  ***** ****
Q H P V L T R I S A A L S L R D A A E L A A L D Q G A E R V

V R E T V E A L R G P G F G E R L G E N Q X
*   *   ***** | | | | ***  *
V L E M V E A L G D A T M D - R L G G N X
```

puf Q *Rb. sphaeroides : Rb. capsulatus*

```
M S D H A V N M P V H A V R A H G H R A S S A E L S V Y F A
              |*|*|   *   *|   *|   |*|   |**|
    M Q S Q R L R A H G V Q - H V D R V P R P E F A L Y F S

V I M L G A F W W L R C S W I V S T I R H R R L P K R G P F
| *| |  *          |*|||  |*  **|*   ***
L I L I V A V P F A L V G W V M A L V R Q R R I P E C G P F

A S A W F D A K A I T P L I F R A X
*  **  |*   *** *  *** |
A R A W R E A G E I T P E I F R P X
```

FIGURE 6.10.

Homology between the deduced amino acid sequences of puf R and puf Q from Rb.sphaeroides and Rb.capsulatus (Bauer et al., 1988). Amino acids conserved in both species are indicated by an asterisk, whereas amino acids sharing similar functions are indicated by a line. X represents a termination codon.

CHAPTER 7

CONSTRUCTION OF A MUTANT OF Rb.SPHAEROIDES LACKING THE LH1 ANTENNA COMPLEX

7.1. INTRODUCTION

The photosynthetic unit of the bacterium Rb.sphaeroides consists of two light harvesting complexes LH1 and LH2 which harvest light and funnel the excitation energy to the reaction centre (Vos et al., 1986; Vos et al., 1988). The light harvesting complexes of this and other bacteria have been classified into two groups depending on their absorbance maxima (Zuber 1986b). The LH1 complex has an absorbance maximum at or around 875nm and LH2 has two absorbance maxima, which in Rb.sphaeroides is 800nm and 850nm. The transfer of energy between pigments is usually down an energy gradient, and occurs from bchl 800 to bchl 850 of LH2 and then to bchl 875 of LH1 which transfers energy to the reaction centre (van Grondelle et al., 1983; Kramer et al., 1984a; van Grondelle and Amesz, 1986). More recently, other pigments have been detected in the antenna of Rb.sphaeroides, namely bchl 870nm (van Dorssen et al., 1988) and bchl 896nm (Bolt et al., 1981; Kramer et al., 1984a; van Grondelle et al., 1988). These are thought to facilitate energy transfer between LH2, LH1 and the reaction centre.

The photosynthetic unit is arranged for efficient energy transfer with a reaction centre/LH1 core of fixed stoichiometry containing about 4 reaction centres and 24 LH1 complexes (6bchl/complex), which are in turn surrounded by a variable "lake" of LH2 complexes (Monger and Parson, 1977; Hunter et al., 1988b; Hunter and van Grondelle, 1988; Vos et al., 1988). The role of LH1 in forming the photosynthetic unit and in energy transfer has been investigated in mutants of Rb.sphaeroides and Rb.capsulatus lacking the LH1 complex (Meinhardt et al., 1985; Jackson et al., 1986,; Jackson et al., 1987; Kiley et al., 1988). The Rb.sphaeroides $LH1^-$ mutant RS103 appears to retain the characteristic ICM vesicles which contain the photosynthetic unit, whereas the $LH2^-$ mutant RS104 does not (Kiley et al., 1988). ICM vesicles are also absent from the $LH2^-$ mutant M21

(Hunter et al., 1988b). The mutant RS103 has a greatly reduced efficiency of energy transfer from LH2 to the reaction centre, and will not grow photosynthetically under low light conditions, whereas RS104 will (Kiley et al., 1988).

Little is known of the features of LH1/reaction centre interactions that promote efficient transfer of excitations to the special pair, although it should be noted that the rate of energy transfer from bchl 896 to the reaction centre is relatively slow, at 50ps (Sundstrom et al., 1986). Nevertheless a "core" of LH1 type antenna is a common feature in photosynthetic bacteria and in a more general sense in cyanobacteria and higher plants. The fitness for LH2 as a donor to the reaction centre could be quite high in view of the similarities between LH1 and LH2 polypeptides (Zuber, 1987), but earlier reconstruction experiments (Hunter et al., 1979c) did not demonstrate fully restored energy transfer in LH2/reaction centre systems. It is therefore important to generate an $LH1^-$ mutant so that it can be compared with the wild type and with the LH1/reaction centre mutant M21. The studies of Meinhardt et al. (1985) and Jackson et al. (1986 and 1987) demonstrate that LH2 does not donate energy in a fully efficient manner to the reaction centre, only 24% of that found in wild type. These studies should be extended to measurements of rates of transfer which can be measured by time resolved picosecond spectroscopy (Sundstrom et al., 1986).

The genes encoding the LH1 α and β apoproteins are encoded by the puf A and B genes and are found as part of the puf operon, which also encodes the reaction centre L and M subunits (Williams et al., 1983). The puf operon has been sequenced (Williams et al., 1983; Williams et al., 1984; Kiley et al., 1987; Chapter 6). This chapter describes the construction of a genetically defined and stable LH1 deficient mutant using the cloned puf operon (Williams et al., 1983) and a puf operon deletion/insertion mutant (Burgess, J.G., personal communication). puf B was mutagenised by cutting at an AccI site in the middle of the gene then filling in the 5' overhangs, a manipulation which causes a 2

basepair insertion, thus changing the open reading frame from 49 to 25 amino acids. Spectral analysis and SDS-PAGE cannot detect the LH1 complex and the LH1 α polypeptide respectively. Northern blot analysis shows the presence of the 2.6kb *puf* B, A, L, M, X transcript but cannot detect the 0.5kb *puf* B,A transcript (Zhu *et al.*, 1986b; Kiley and Kaplan, 1988; Chapter 4; Chapter 6).

7.2. RESULTS

The construction of the LH1 deficient mutant is illustrated in figure 1. The 6.5kb *Eco*RI-*Hind*III fragment containing the *puf* operon was subcloned from pJW1 (Williams *et al.*, 1983) into Bluescribe+ and the construction was denoted pBSEH1. The genes encoded by the *puf* operon are indicated on the map (Williams *et al.*, 1983; Kiley and Kaplan, 1988; Chapter 6). A 2.45kb *Eco*RI-*Sal*I fragment was subcloned into Bluescribe+, denoted pBSES1. The sequence of this *Eco*RI-*Sal*I fragment is known (Williams *et al.*, 1984; Kiley *et al.*, 1987; Chapter 6), and contains four *Acc*I sites. One of these *Acc*I sites occurs halfway through the *puf* B gene. This enzyme produces a staggered cut with a 2 base 5' overhang, which can be filled in with Klenow and the four deoxynucleotidetriphosphates, thus destroying the *Acc*I recognition site. If the *Acc*I site in the *puf*B gene is filled in, a 2 basepair insertion occurs, shifting the reading frame of the *puf*B gene downstream of the *Acc*I site. A stop codon then occurs in the reading frame just downstream of the *Acc*I site changing the encoded polypeptide from 49 to 25 amino acids.

This mutagenesis was performed by purifying single cut pBSES1 from an *Acc*I partial digest, checked by comigration with *Eco*RI cut pBSES1. The purified fragment was filled in with Klenow and dNTP's and then blunt end ligated and transformed into *E.coli* DH5. Plasmid DNA prepared from ampicillin resistant colonies was digested with *Acc*I in order to determine which one of the four *Acc*I sites had been lost. When digested with *Acc*I the wild type clone pBSESI produces fragments of 302bp, 574bp and 582bp (which appear as a doublet) and 4kb. When the *Acc*I site in the *puf*B gene is 'filled in' the 302bp and 574bp fragments are lost and an

878bp fragment is produced. As a result of screening 24 'mini plasmid' preparations the desired clone, pBSESAI was identified. In order to check that the AccI site had been lost by a 2 basepair insertion, and not by degradation of the 5' overhangs, the 518bp AluI fragment containing the pufB gene (chapter 6) was subcloned from pBSESA1 into M13 mp18 and sequenced. The sequence of the AluI fragment from pBSESA1 only differed from the wild type AluI fragment (Kiley et al., 1987; Chapter 6) by the insertion of the sequence TC where the AccI site in the pufB gene was originally located.

The puf operon was reconstructed by digesting pBSEH1 with EcoRI and then partially with SalI. The 6.0kb fragment was purified which had lost the 2.45kb EcoRI-SalI fragment. The 2.45kb EcoRI-SalI fragment from pBSESA1 was subcloned into this to reconstruct the mutated puf operon in the construct pBSEHA1.

The construct pBSEHA1 was transferred into the puf operon deletion strain DPF1 (Burgess, J.G. personal communication) by conjugal plasmid transfer from E.coli Sm10. Transconjugants were selected by photosynthetic growth on M22+. Three slowly growing red colonies were obtained, MA1, MA3 and MA5, which in addition to being photosynthetic, had also lost kanamycin resistance by exchange of the mutated puf operon with the Km^r gene and had absorbance spectra characteristic of the LH1 deficient mutants already published (Meinhardt et al., 1985; Jackson et al., 1986; Jackson et al., 1987).

The absorbance spectra of membranes purified from wild type Rb.sphaeroides, MA3 and DPF1 are shown in figure 2. Wild type Rb.sphaeroides (figure 2c) has the peaks at 800nm and 850nm characteristic of LH2 and a shoulder at 875nm due to LH1. The puf operon deletion/insertion mutant DPF1 shown in figure 2A exhibits no absorbance in the near infrared indicating the absence of LH1, LH2 and the reaction centre (see chapter 6). In strain MA3 absorbance peaks at 800nm and 850nm are restored due to the transfer of pufR and pufQ to DPFI (see chapter 6). The heights of the peaks are now nearly equal however, whereas in the

wild type the 850nm peak is twice the height of the 800nm peak due to the presence of two bchl 850 molecules per bchl 800 molecule (Kramer et al., 1984b). There appears to be more than one spectral component in the 850nm peak indicated by the half peak width of this peak in comparison to the 800 nm peak. Spectra similar to this have been presented for LH1 deficient mutants of Rb.sphaeroides and Rb.capsulatus (Meinhardt et al., 1985; Jackson et al., 1986; Jackson et al., 1987).

Figure 3 shows the absorbance spectra of membranes purified from the LH1 only mutant NF57 and MA3. In addition to the characteristics discussed in figure 2 it can be seen that the 800nm peak of MA3 is shifted 5nm to the red relative to NF57 and the 850nm peak is shifted 9nm to the red relative to NF57.

Figure 4 shows SDS-PAGE analysis of membranes purified from wild type Rb.sphaeroides DPF1 and MA3. The reaction centre H, M and L subunits and light harvesting polypeptides are labelled. The light harvesting bands are observed in SDS-PAGE, which are LH2 α, LH1 α and comigrating LH1 and LH2 β polypeptides in order of apparent decreasing molecular weight (Broglie et al., 1980). None of these polypeptides are detectable in DPF1 membranes (track B). Track C shows MA3 membranes. The LH1 α polypeptide is not detectable, and the third LH polypeptide band appears to have the same intensity as the LH2 α band, suggesting that the LH1 β polypeptide may be absent. A large number of other polypeptides are present in track C. This is because the sonicated cells were loaded directly onto 40% sucrose rather than a 40% and 15% sucrose step gradient. The normal procedure had been modified to take account of the possibility that the ICM vesicles in MA3 may be less dense than wild type vesicles due to the reduced amount of light harvesting pigment (Niederman et al., 1981). The fraction obtained under this procedure, however appears to contain a number of ribosomal and cell wall proteins which have remained at the top of the gradient. Further purification will have to be undertaken to remove these proteins.

Total RNA was prepared from MA3 24 hours after a culture of this strain had been transferred from aerobic to photosynthetic growth conditions and then analysed by Northern blotting with the 518bp AluI fragment containing the pufB and A genes used as a probe (figure 5). Track A shows wild type RNA prepared 2 hours after the onset of pigment indiction (Niederman et al., 1979) showing the bands at 2.6kb, 1.15kb, 0.61kb and 0.48kb as described in chapter 6. Track B shows MA3 RNA with two bands at 2.6kb and 1.3kb. It appears that the 1.15kb, 0.61kb and 0.48kb transcripts are absent in mutant strain MA3. The 0.48kb transcript is the most abundant mRNA species, and encodes pufB and A (Zhu et al., 1986b; Chapter 4; Chapter 6); it appears to be destablised by the mutation in the puf B gene of strain MA3. Unfortunately these two RNA samples are not strictly comparable as they have been prepared from cells under different growth conditions. RNA will have to be prepared from MA3 undergoing pigment induction.

7.3. DISCUSSION

In this study I have constructed an LH1 deficient mutant by introducing a known and stable mutation into the pufB gene. An NTG mutant deficient in LH1, which would have arisen from a point mutation would be able to revert at a low frequency. Unfortunately, revertants would grow faster than the mutants in photosynthetic conditions, quickly outgrowing them in the culture, as has been found for mutant M21 (unpublished data; Niederman, R.A., personal communication). The mutation in the pufB gene changes the β polypeptide from 49 to 25 amino acids, removing half of the hydrophobic transmembrane domain which includes the histidine residue (His - 38) involved in binding bchl as well as all of the C terminal polar charged domain (Zuber, 1987). Bylina et al., (1986) have shown in Rb.capsulatus that by merely changing the histidine residue (His-32) of the LH1 polypeptide to other amino acids completely destablizes the LH1 complex. This would indicate that truncation of the LH1β polypeptide should also completely destablize the LH1 complex.

The mutation was made in this way rather than by mutating the pufB and A genes with a transposon or interposon because the pufL

and M genes are transcriptionally linked, downstream of the pufB and A genes (Zhu et al., 1986b) as are the pufQ and R genes upstream of pufB (Bauer et al., 1988; Chapter 6). Transposon or interposon insertions in the pufB and A genes would stop further downstream transcription of the pufL and M genes and might affect transcription of the pufQ and R genes.

Northern blot analysis of total RNA from MA3 shows that the 2.6kb transcript encoding puf B, A, L, M and X is present whereas the 0.5kb transcript encoding pufB and A, which is normally present at higher levels than the 2.6kb transcript in the wild type (Zhu et al., 1986b) cannot be detected. A 1.3kb transcript which has not been observed before was also present, and may be an intermediate breakdown product of the 2.6kb transcript (see chapter 6). The absence of some transcripts and the presence of a new transcript may be linked in some manner to the assembly and turnover of LH1 polypeptides, although the mechanism of its regulation are not known.

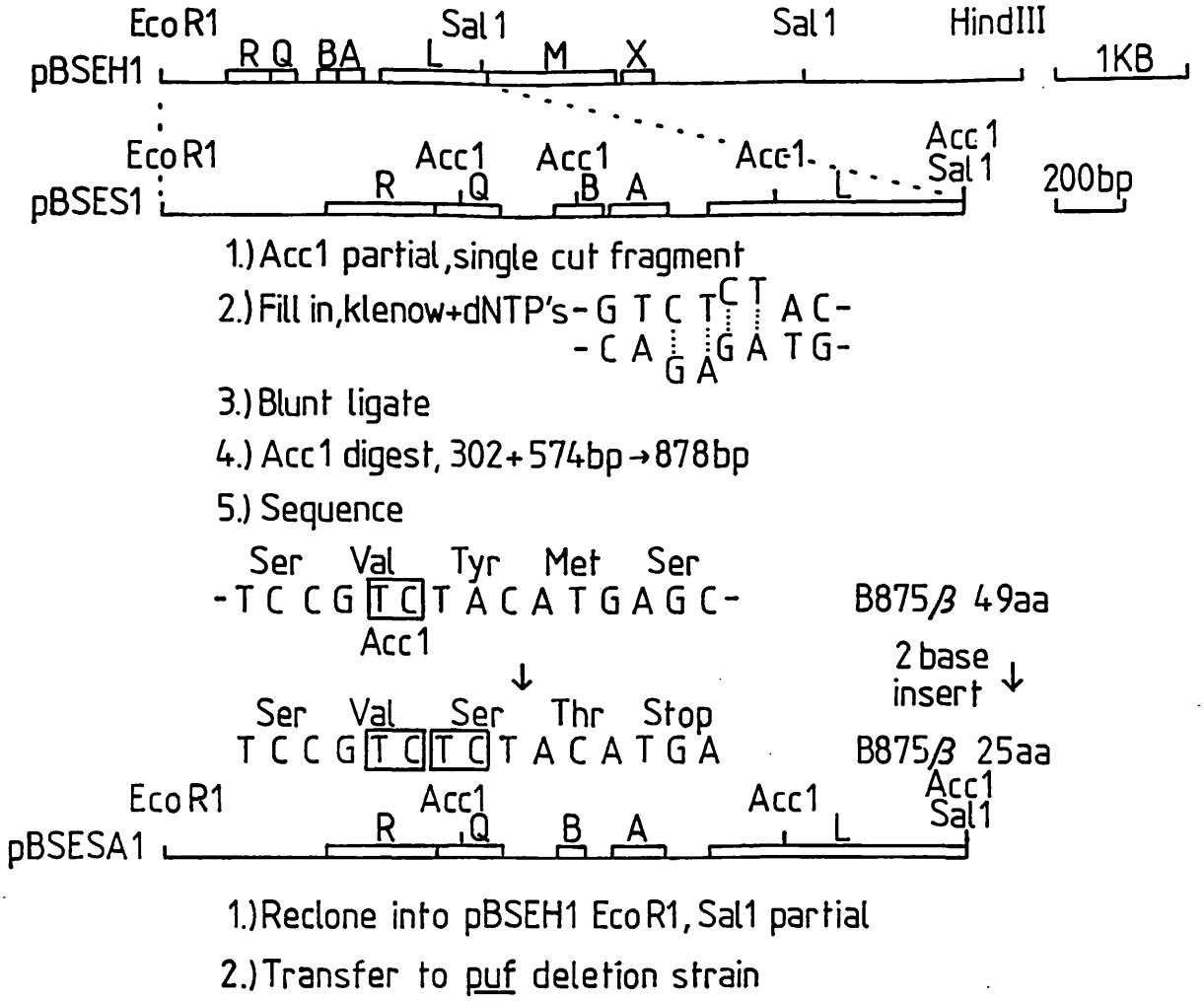
The spectra of MA3 shows that LH2 has been restored to DPF1, presumably due to the cotransfer of pufR and Q (chapter 6). The ratio of the 800nm to 850nm peaks is different to that in wild type (Kramer et al., 1986b) and in mutant NF57 (Hunter and van Grondelle, 1988) as is the half peak bandwidth. This would suggest that there are other spectral components in addition to bchl 850 which modify the absorbance profile. The special pair bateriochlorophyll P870 of the reaction centre may absorb to a significant extent in this mutant, and also possibly the 870nm absorption of some of the LH2 bchl's which have been found in NF57, a mutant containing LH2 as the only pigment protein complex (Vos et al., 1988; van Dorssen et al., 1988). The 800nm and 850nm peaks have also been shifted slightly to the red compared to NF57, indicating that the reaction centres are influencing LH2 absorption.

The SDS-PAGE analysis confirms that the LH1 complex is absent or greatly reduced, as the LH1 α polypeptide is undetectable. The LH1 α and β polypeptides are probably still made, but would be

rapidly degraded because a stable bacteriochlorophyll-protein complex cannot be formed because pigment binding sites in the polypeptide as well as possibly important structural sequences have been removed.

This mutant awaits further characterisation by Southern blot analysis to confirm that the puf operon has integrated into the chromosome by a double homologous recombination events between the sections of DNA flanking the kanamycin cassette in the chromosome of DPF1 and the construct pBSEHA1 a process which would remove the kanamycin cassette as described in chapter 5. This event would also prevent the cloning vector for pBSEHA1 from integrating into the chromosome.

Further work on this mutant would involve low temperature singlet-singlet annihilation and time resolved picosecond absorption recovery to ascertain the size of the photosynthetic unit, the efficiency of energy transfer from LH2 to the reaction centre and to determine the presence of any minor bchl species in LH2. The ultimate aim with this mutant is to construct a mutant with the reaction centre as the sole pigment protein complex by using the construct pMSK11 to delete the puc operon from the chromosome (as described in chapter 5). Thus for the first time it would be possible to study the properties of pure reaction centres in the native phospholipid membrane, without the possible detrimental influence of detergents on this complex.



1.) Reclone into pBSEH1 EcoR1, Sal1 partial

2.) Transfer to puf deletion strain

Figure 7.1.

Construction of a mutant of Rb.sphaeroides deficient in LH1.

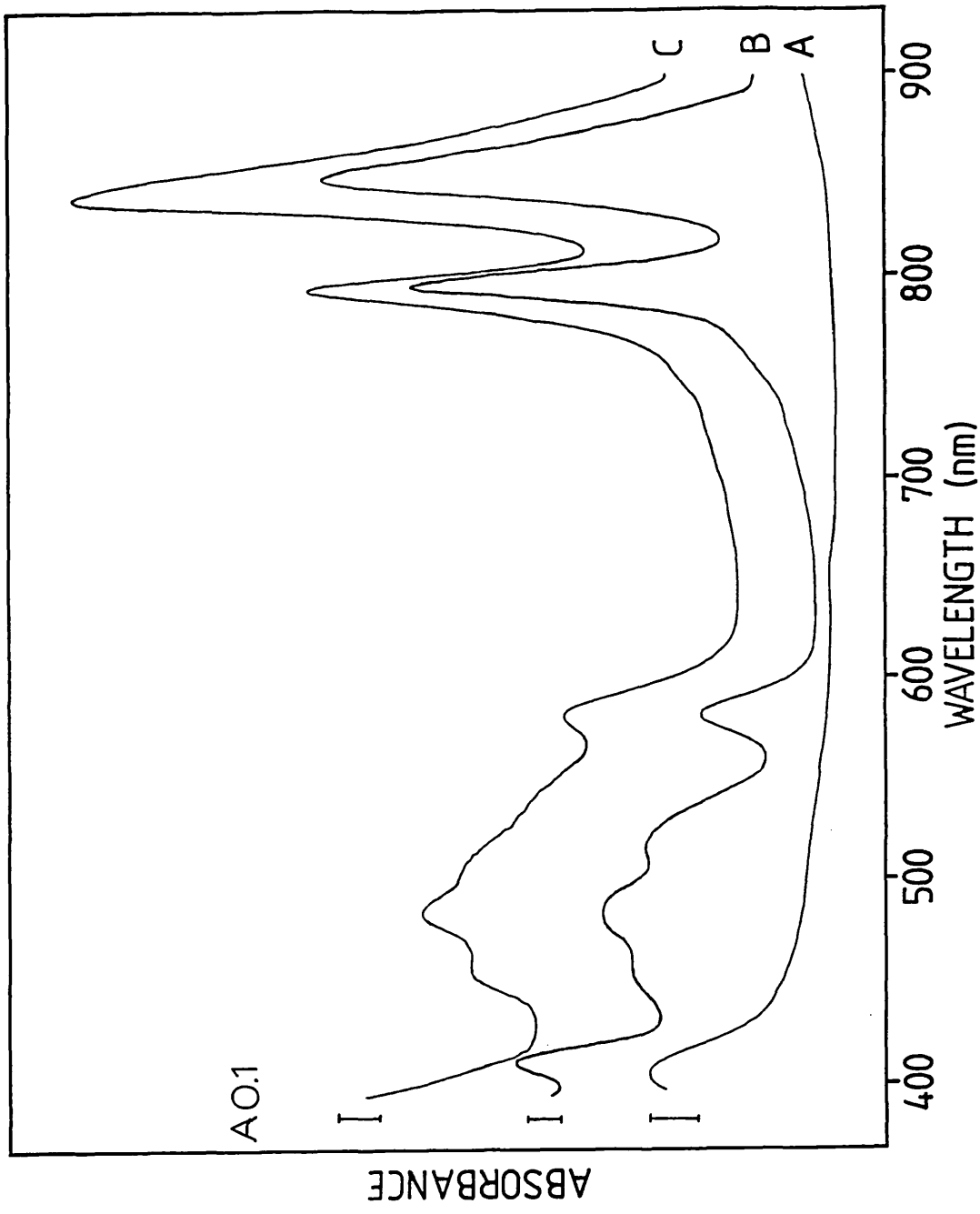


Figure 7.2.

Absorbance spectra of membranes purified from:
A. DPF1; B. MA3; C. Wild type Rb.sphaeroides.

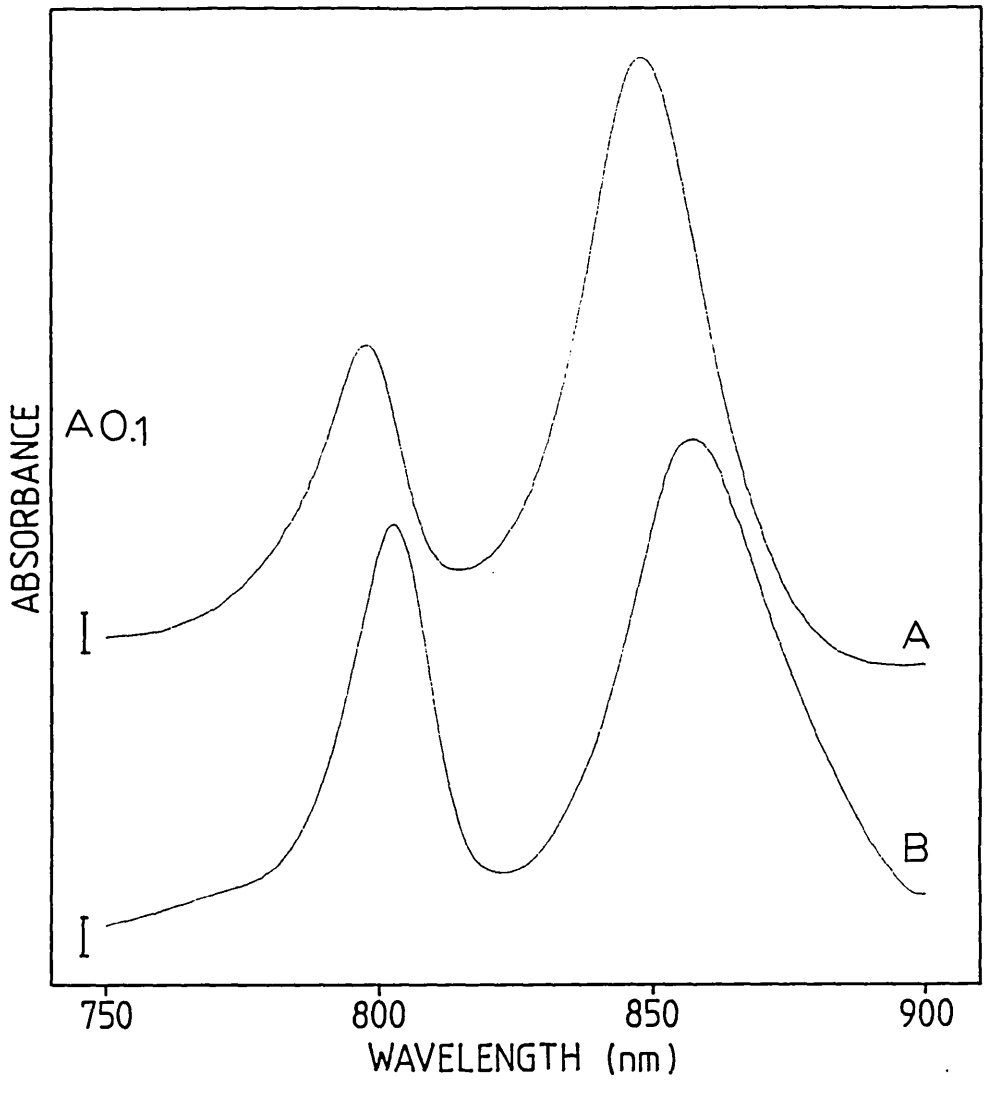


Figure 7.3.

Absorbance spectra of membranes purified from:
A. NF57; B. MA3.

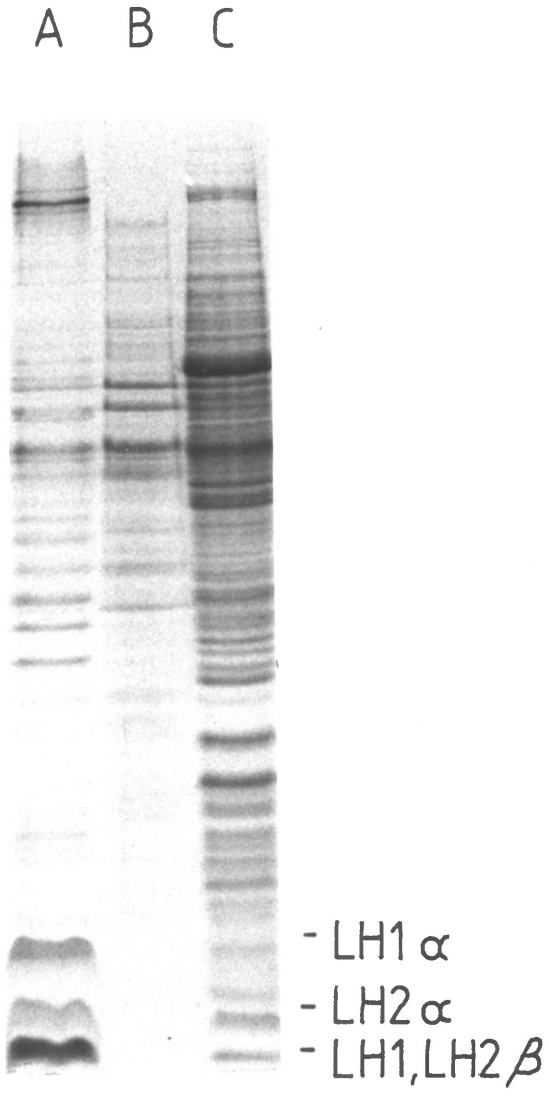


Figure 7.4.

SDS-PAGE analysis of membranes purified from:
A. Wild type Rb.sphaeroides; B. DPF1; C. MA3.

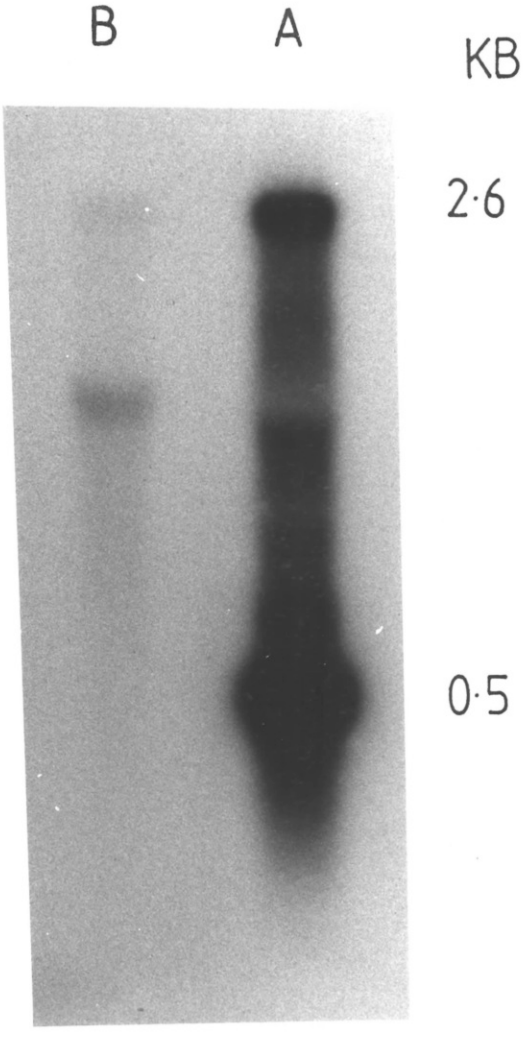


Figure 7.5.

Northern blot analysis total RNA prepared from:
A. Wild type Rb.sphaeroides: B. MA3. The blot was hybridised with a ^{32}P labelled AluI fragment containing the pufB and A genes.

CHAPTER 8

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