# Peripheral Antenna Complexes from *Rhodopseudomonas acidophila*: Structure, Function and Genetic Manipulation.

by

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## Declaration.

This thesis is an original composition which describes work performed entirely by myself unless otherwise cited or acknowledged. Its contents have not previously been submitted for any other degree. The research for this thesis was performed between October 1987 and February 1992.

Signed..

Alastair T. Gardiner

Date: 26th November 1992.

## Summary

All members of the Rhodospirillaceae adapt to a reduced level of light available for photosynthesis by synthesising a greater amount of intra-cytoplasmic membrane (ICM) per cell and, wherever possible, increasing the number and size of photosynthetic units (PSU). It was already known that certain strains of *Rhodopseudomonas acidophila* have a further adaptative response (Cogdell *et al.*, 1983), in that they are able to alter the composition of their PSU by replacing the peripheral B800-850 complex with B800-820. This work delineates the relationship between these two complexes with respect to the light-harvesting capability of this species when grown under different (non-optimal) conditions and sets up a genetic system to introduce cloned DNA into Rhodospirillaceae, enabling the molecular organisation of these genes and their regulation to be investigated.

As a prerequisite to any molecular genetic experiments a thorough characterisation of the conditions under which B800-820 is inserted into the ICM was necessary. Three wild type isolates of *Rps. acidophila* were cultured under three different growth regimes: (1) progressively decreasing light intensity, (2) progressively decreasing temperature at high light, and (3) progressively decreasing temperature at low light. Chromatophores prepared from each series were analysed using absorption spectroscopy, Deriphat-PAGE and reversed phase HPLC. The conclusions reached concerning each of the strains are as follows:

***Rps. acidophila* 7750**: This strain produces B800-820 complex in response to low light and low temperature, however, the light intensity 'switch' takes precedence over its temperature counterpart. No evidence was obtained from Deriphat-PAGE that there was any change in the B890/Reaction Centre (RC) conjugate, specifically, in its carotenoid composition; HPLC revealed that the low light B800-820 complex contains relatively more unglucosylated rhodopin than the high light B800-850 complex.

***Rps. acidophila* 7050**: This strain produces B800-820 complex in response to low light; no evidence could be found of any temperature regulation. From Deriphat-

PAGE, the carotenoid composition of both the peripheral and fixed complexes altered under low light. The main change in the peripheral antenna was the replacement of orange coloured rhodopin and its glucoside with purple rhodopinal glucoside. Furthermore, this carotenoid change was not synchronous with, rather preceded, the switch in the peripheral antenna.

***Rps. acidophila* 10050**: No evidence was obtained that this strain was able to produce B800-820 under any of the conditions tried. Furthermore, the carotenoid compositions of the peripheral and fixed complexes also remained unchanged.

Data was then presented to explain, with regard to the light-harvesting capability of the cell, the insertion of B800-820 in place of B800-850 when the cell is cultured at non-optimum conditions. Fluorescence emission experiments showed that once excitons have arrived in B890 they can be transferred back to B800-850 but not to B800-820. Singlet-singlet annihilation experiments revealed a B890 domain size of approximately 50 ± 20 Bchls when the peripheral antenna was B800-820, and at least 200 for the corresponding measurement with B800-850. Thus, B800-820 is produced at non-optimum conditions because it is more efficient at funnelling excitons into B890 and on to the RC.

A genetic system based on bacterial conjugation (Simon *et al.,* 1983) was then developed which allowed the introduction of cloned DNA into *Rps. acidophila,* and two other species with interesting peripheral antennae, *Rps. palustris* and *R. rubrum.* The results proved that Inc-P and Inc-Q cloning vectors could be mobilised into these species and that transposon mutagenesis was also feasible.

An EMBL3 genomic library had previously been constructed from *Rps. acidophila* 7050 DNA and a preliminary attempt made to characterise clones that contained peripheral antenna complex genes (M$^{ac}$Kenzie, 1990). One of these clones, λ6, was known to carry a β/α gene pair on a 2.1kb Sall fragment. This fragment was sequenced and found to contain not one but three β/α gene pairs, designated (5 → 3') sxaBA, sxbBA and sxcBA, which when translated were exceedingly homologous to the α, $\beta_1$ and $\beta_2$ polypeptides from strain 7750.

To verify that the above system to introduce cloned DNA was feasible in an experimental context, the 2.1kb SalI fragment was cloned into a mobilisable vector and introduced into *R. rubrum.* No constitutive expression of any of the genes from this cluster was apparent, although there was a carotenoid pleiotropic effect which was not able to be further investigated. This preliminary experiment, nevertheless, established the feasibility of using this system in the future to investigate at a molecular genetic level the organisation and regulation of peripheral antenna genes from *Rps. acidophila.*

## Acknowledgements.

I would like to take this opportunity to thank the many people with whom I have come into contact during my time in the Dept. of Botany. My interest, indeed love of science and the altruistic principle behind it, has been fostered by numerous wonderful people, too many to mention individually, in the Department and elsewhere that I have had the privilege to know in the time I have been working on this thesis.

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## Abbreviations.

Unless otherwise stated S.I. units have been used.

| | |
|---|---|
| Å | Angstrom ($10^{-8}$m). |
| AHRV | Anhydrorhodovibrin. |
| Ap | Ampicillin. |
| A. U. | Arbitrary units |
| Ab. U. | Absorbance units |
| Bchl | Bacteriochlorophyll. |
| BOG | n-ß-octyl Glucoside. |
| bp | Base pairs. |
| Bpheo | Bacteriopheophytin. |
| 'closed traps' | Reaction centre primary electron donor (P-870) is oxidised. |
| DAD | Diode array detector |
| DDM | n-dodecyl-ß-D-Maltoside. |
| Deriphat 160 | N-lauryl-ß-iminopropionate. |
| EMBL3 | Bacteriophage λ replacement vector. |
| eV | Electron volt. |
| ExoIII | Exonuclease III. |
| HL | High light cultured cells. |
| HPLC | High performance liquid chromatography. |
| ICM | Intra-cytoplasmic membrane. |
| Inc-P, Q | Plasmid belonging to incompatibility group P or Q. |
| kb | kilo Base pairs. |
| kD | kilo Dalton. |
| Km | Kanamycin. |
| LH | Light-harvesting. |
| LL | Low light cultured cells. |
| LT | Low temperature and light cultured cells. |
| Mob | Genetic locus that acts as a recognition site for tra. |
| MES | 2-(N-Morpholino) ethanesulphonic acid. |
| NADH/NAD$^+$ | Reduced/Oxidised Nicotinamide Adenine Dinucleotide. |
| N | number of conjugated double bonds |
| n. d. | not determined. |
| NIR | Near infra-red. |
| Nm | Neomycin. |
| ODS | Octadecylsilyl. |
| 'open traps' | Reaction centre primary electron donor (P-870) is reduced. |
| ORF | open reading frame. |
| PAGE | Polyacrylamide gel electrophoresis. |
| PCR | Polymerase chain reaction. |
| PSU | Photosynthetic unit. |
| P-870 | Bacteriochlorophyll primary electron donor in the *Rps. acidophila* reaction centre. |
| $Q_x$, $Q_y$ | Bacteriochlorophyll porphyrin ring absorption dipoles in the x- and y-planes respectively. |

| | |
|---|---|
| r | Annihilation parameter. |
| RC | Reaction centre. |
| RE | restriction endonuclease. |
| RF | replicative form. |
| RT | room temperature. |
| SD | Shine-Dalgarno ribosome binding sequence. |
| SDS | Sodium dodecyl sulphate. |
| Sm | Streptomycin. |
| tra | *trans*-acting transfer functions necessary for conjugation. |
| Tc | Tetracycline. |
| UV-CD | Ultra violet-circular dichroism. |

The IUPAC-IUB one-letter amino acid code has been used throughout this thesis.

| | |
|---|---|
| A | Alanine. |
| C | Cysteine. |
| D | Aspartate. |
| E | Glutamate. |
| F | Phenylalanine. |
| G | Glycine. |
| H | Histidine. |
| I | Isoleucine. |
| K | Lysine. |
| L | Leucine. |
| M | Methionine. |
| N | Asparagine. |
| P | Proline. |
| Q | Glutamine. |
| R | Arginine. |
| S | Serine. |
| T | Threonine. |
| V | Valine. |
| W | Tryptophan. |
| Y | Tyrosine. |

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# Chapter One

# Introduction

## 1.1 An overview of prokaryotic photosynthesis.

The photochemical reduction of $CO_2$ to organic substrates, which are then used to maintain the organisation and growth of the organism, is called photosynthesis. This energy transduction reaction is central to life as it is the ultimate source of food for almost all organisms and its scale is vast: on the Earth some $2x10^{11}$ tonnes of carbon are fixed each year.

The process is limited to a few genera of photosynthetic bacteria, cyanobacteria, various eukaryotic algae and the higher green plants: even so mechanistic differences were apparent at a very early stage. Consider the overall equation (1) for formation of glucose by photosynthesis in higher plants:

$$6CO_2 + 6H_2O \xrightarrow{h\nu} 6O_2 + C_6H_{12}O_6 \qquad (1)$$

The stoichiometry of equation (1) suggests that all 12 of the oxygen atoms of the evolved $O_2$ might have come from $CO_2$ or that some originated from $CO_2$ and some from $H_2O$. In fact it is now known that water supplies all of the oxygen atoms for formation of $O_2$ as in equation (2)

$$6CO_2 + 12H_2O^* \longrightarrow 6O_2^* + C_6H_{12}O_6 + 6H_2O \qquad (2)$$

This idea was first suggested by C.B. van Niel in 1933. He pointed out that in bacterial photosynthesis no $O_2$ is produced and that bacteria must have access to a reducing agent to provide hydrogen for the reduction of $CO_2$ as in equation (3)

$$H_2A + \text{light} \longrightarrow A + 2[H] \qquad (3)$$

In this equation, $H_2A$ might be $H_2S$ (as in the purple sulphur bacteria), succinate (purple non-sulphur bacteria), elemental $H_2$, etc. From a consideration of these various reactions, van Neil reached the logical conclusion that in the $O_2$-

producing blue-green algae (cyanobacteria) and higher plants water serves as the oxidisable substrate in equation (3). Thus, $H_2O$ is cleaved to form $O_2$ and to provide hydrogen atoms for reduction. It is interesting to note that this is the only known biological oxidation of $H_2O$. No oxidising agents present in living matter are powerful enough to dehydrogenate water except for the photosystems present in the oxygen evolving photosynthetic organisms.

From this elementary principle further research, whilst increasing our knowledge of the nature of photosynthesis, has also revealed the extremely complex structure of the photosynthetic membrane and the mechanisms involved that underpin its function.

This complexity is so sophisticated that a co-ordinated understanding of the processes involved in photosynthesis encompassing e.g., primary photochemistry, redox chemistry, bioenergetics and protein structure/function as well as the regulation of the numerous genes involved that pertain to assembly of the photosynthetic membrane and its components, will not be possible without recourse to phototrophic bacteria. These bacteria are widely used in photosynthetic research, partly as they are intrinsically interesting and important in their own right but also because broadly parallel processes occur in photosynthetic bacteria as in higher plants. These are very much simpler, hence more amenable to study through biochemical and molecular biological techniques and so insights gained into bacterial mechanisms can be used to derive a similar degree of understanding from their higher plant counterparts which would otherwise not have been possible. Thus, the study of photosynthetic bacteria is crucial to developing an overall understanding of this fascinating and intriguing process.

## 1.2 Taxonomy of the Rhodospirillales.

Phototrophic bacteria are classified in the order Rhodospirillales, which is defined as comprising those bacteria that contain bacteriochlorophylls (Bchls) and carry out anoxygenic photosynthesis. Figure 1.1 outlines the current taxonomy of

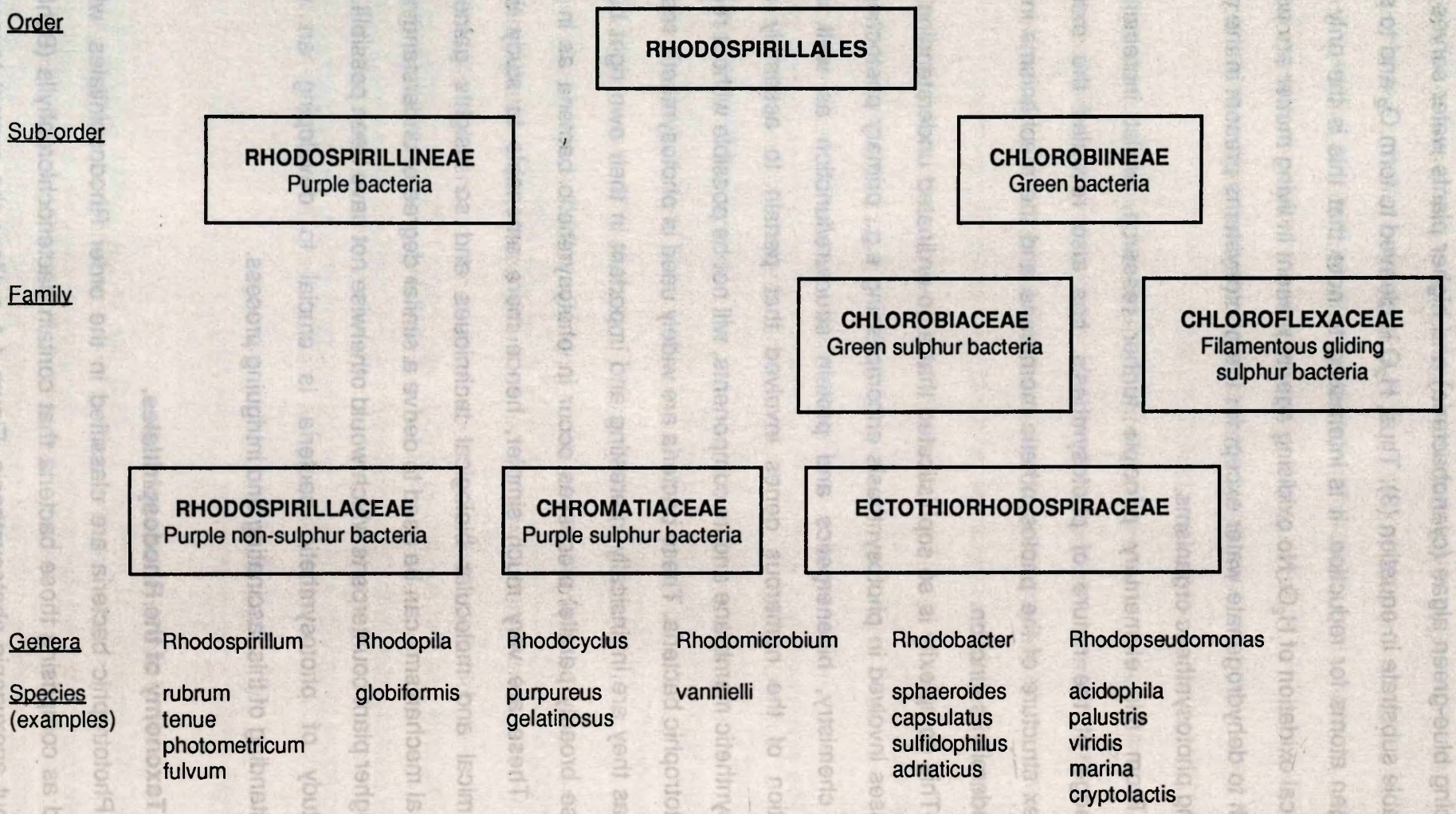



**Figure 1.1:** Current classification of the phototrophic bacteria. Only genera and species from the Rhodospirillaceae are shown.

the Rhodospirillales, which is divided into two sub-orders, the Rhodospirillineae and the Chlorobiineae.

The sub-order Chlorobiineae comprises those phototrophic bacteria that contain Bchl c, d or e. These pigments are located in non-unit-membrane bound lens- to cigar-shaped organelles called chlorosomes which underlie the cytoplasmic membrane. This sub-order contains the families Chlorobiaceae and Chloroflexaceae.

The sub-order Rhodospirillineae comprises those bacteria that contain only Bchl a or b; these pigments are always located in intra-cytoplasmic membrane (ICM) systems of different types continuous with the cytoplasmic membrane. This sub-order comprises the families Rhodospirillaceae, Chromatiaceae and Ectothiorhodospiraceae.

The purple sulphur bacteria (Chromatiaceae) grow well under photoautotrophic conditions. All of them can grow with sulphide and some representatives also use elemental sulphur, sulphite or thiosulphate as electron donors. During oxidation of sulphide to sulphate elemental sulphur is deposited inside the cells. Photoheterotrophic, chemoautotrophic and/or chemoheterotrophic growth has been demonstrated for several species. Various types of carotenoids and, in most species, Bchl a serve as photosynthetic pigments. These are located in ICM that are formed as vesicles in all species except *Thiocapsa pfennigii.*

The Ectothiorhodospiraceae are distinguished from the Chromatiaceae by the intermediate disposition of elemental sulphur outside the cells. There is a characteristic dependence on saline and alkaline growth conditions. ICM are present as lamellar stacks.

The purple non-sulphur bacteria (Rhodospirillaceae) are the most diverse and best studied group of phototrophic bacteria. These bacteria grow preferably under photoheterotrophic conditions and many representatives are aerotolerant and can grow as chemoheterotrophs in the dark. Moreover the ability in some members of this family to fix atmospheric nitrogen permits an integrated study of these

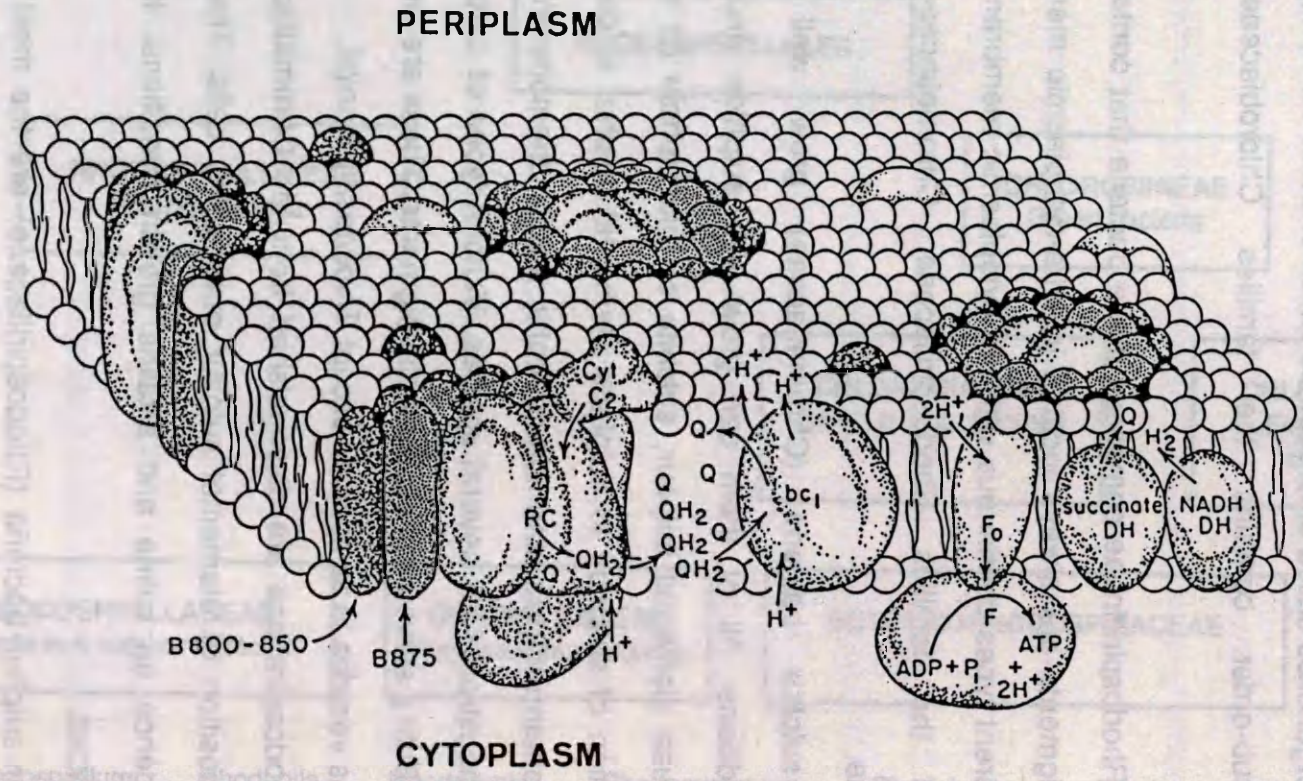



**Figure 1.2:** Schematic illustration of a section through the *Rb. sphaeroides* ICM bilayer depicting the relative orientation of the specific components. Reproduced from Kiley and Kaplan, (1988).

diverse metabolic activities often separated in other biological systems. The results presented in this study have been obtained from experiments performed on members of this family.

## 1.3 ICM Structure and function in the Rhodospirillaceae.

Imhoff *et al.*, (1984) recommended a rearrangement of the species and genera of the Rhodospirillaceae to better reflect current data on the inter-relatedness of this group. The most significant change suggested was the removal of all species with vesicular ICM from the genus *Rhodospeudomonas* and to place them in two new genera *Rhodobacter* and *Rhodopila.* This left within the *Rhodospeudomonas* genus, species that contained only ICM that are adjacent and parallel to the cytoplasmic membrane i.e., lamellae.

When growing chemoheterotrophically members of the Rhodospirillaceae have a typical gram-negative cell envelope and growth is supported by aerobic respiration. When oxygen is removed from such a culture, a series of events is triggered which results in the differentiation of the cytoplasmic membrane (CM) through a process of invagination into specialised domains which comprise the ICM. The ICM is physically continuous with the CM but structurally and functionally distinct in that the ICM specifically contains all of the membrane components required for the light reactions of photosynthesis. A schematic view of the ICM is illustrated in Figure 1.2.

The most abundant protein complexes are the Bchl and carotenoid containing light harvesting (LH) complexes that have been designated B875 or B890 (formerly LHI) and B800-850 (formerly LHII), based on their near infrared (NIR) absorption maxima. As their name implies, the LH complexes act as antenna to funnel energy into the reaction centre (RC); Bchl-protein complexes in which this energy is converted to chemical energy by photoinduced oxidation-reduction reactions. The funnelling of photons to the RC occurs in the form of mobile electronic excited singlet ($S_1$) states (excitons), rather than light energy emission

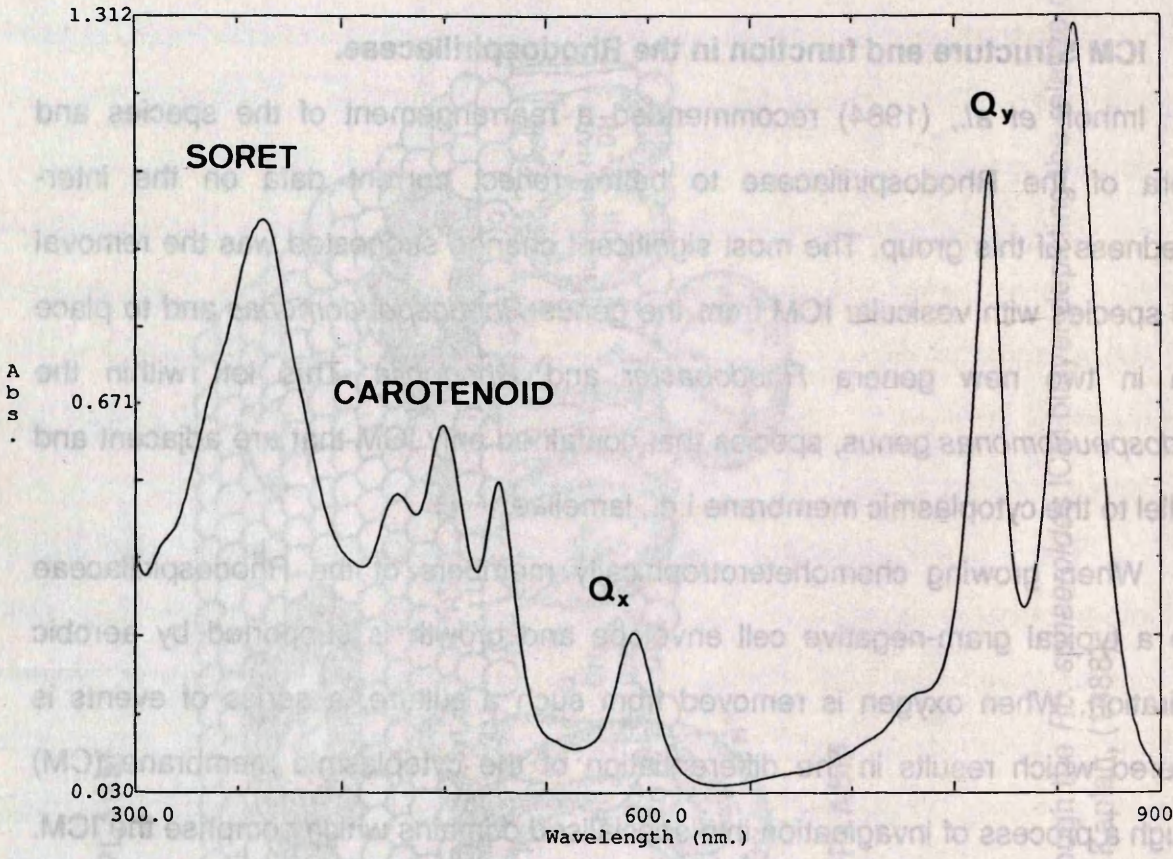



**Figure 1.3:** Absorption spectrum for photoheterotrophically growing *Rb. sphaeroides*. Within this wavelength range the ICM proteins do not absorb light, rather it is the bound pigments which give rise to the characteristic profile. From this spectrum the evolutionary adaptations that avoid direct competition with higher plants for the available light are apparent: the carotenoids absorb in the green visible region, whereas from approx. 600 to 800nm i.e., red wavelengths, there is no absorpton. To compensate, these bacteria extend light-harvesting into the near infra-red.

and reabsorption. This exciton transfer process allows energy migration within the antenna system and to the RC to proceed between pigment molecules by very fast random walk lasting in the order of $10^{-11}$-$10^{-10}$s (van Grondelle, 1985). The arrangements of these Bchl-protein complexes in the ICM must be highly ordered to achieve the high efficiency of exciton transfer, since little of the energy is emitted as fluorescence from wild-type strains during active photosynthetic growth. In a species such as *Rb. sphaeroides* that contains both B875 and B800-850 LH complexes, the B875 complex appears to be an obligatory intermediate in exciton energy transfer from the peripheral B800-850 complex to the RC. Thus, a concentric circular arrangement of the antenna complexes forms a system for heterogeneous energy transfer from the 'outside' of the PSU to the B890 complex and hence to the RC (Zuber, 1986a). Aggregates of B875 surround and possibly interconnect RCs within the ICM: these aggregates have been termed the B875/RC conjugate or the fixed photosynthetic unit since the ratio is invariant at approximately 24:1 Bchls per RC. Direct evidence for a symmetrical arrangement of LH complexes surrounding the RC has been demonstrated only in *Rps. viridis* by immunoelectron microscopy combined with image enhancement (Stark *et al.,* 1986). Extrapolation of biophysical measurements suggest a similar arrangement in other Rhodospirillaceae (see e.g., Drews *et al.,* 1983). The amount of the peripheral B800-850 complex present varies inversely with incident light intensity, (this aspect will be expounded in section 1.4), enabling the cell to adapt to changing environmental conditions. A typical absorption spectrum for *Rb. sphaeroides* is given in Figure 1.3.

The absorption of light energy i.e., the exciton, by the RC ultimately results in the photo-oxidation of a 'special pair' of Bchl molecules (Deisenhofer *et al.,* 1985). The electron is then transferred in turn to another Bchl *a* and on to a bacteriopheophytin (Bpheo) molecule, which then reduces a quinone molecule ($Q_a$) which is in close proximity to an inorganic iron. From $Q_a$ the electron is transferred to another quinone, $Q_b$, which diffuses from its binding site in the RC complex and

equilibrates with the membrane-soluble quinone pool, thereby carrying an electron to the ubiquinol cytochrome $\underline{c}_2$ : oxidoreductase (cyt $\underline{b}/\underline{c}_1$) complex. Cytochrome $\underline{c}_2$ (cyt $\underline{c}_2$) functions as a mobile periplasmic redox carrier in cyclic photosynthetic electron flow by transferring an electron from the membrane bound cyt $\underline{b}/\underline{c}_1$ complex to reduce the photooxidised RC complex. Concomitant with this cyclic electron flow is the vectorial transport of protons from the cytoplasmic to the periplasmic membrane face, through the cyt $\underline{b}/\underline{c}_1$ complex, resulting in an electrochemical proton potential that is coupled to photophosphorylation i.e., ATP synthesis. When succinate is used as the carbon source it is oxidised to fumarate by a membrane bound dehydrogenase complex. This is coupled with the reduction of the membrane quinone pool to enable electrons to feed into either cyclic electron flow, or the $NAD^+$ reduction pathway. Fumarate can then be utilised in carbon metabolism.

## 1.4 Effect of light intensity on the Rhodospirillaceae.

The synthesis of the antenna and RC complexes is not light-dependent, it can proceed albeit to a much lesser extent in the dark without oxygen, rather light intensity is an external signal which regulates the formation of the photosynthetic apparatus under phototrophic growth conditions. All photosynthetic organisms have developed mechanisms of adaptation to different light intensities (Zuber and Brunisholz, 1991; and references therein). An excess of absorbed light can be dangerous if not channelled to the RCs or re-emitted as fluorescence. A shortage of light energy reduces rates of growth and metabolism and diminishes the capacity for competition in the ecological niche.

When exponentially growing cells of e.g., *Rb. sphaeroides* are subjected to a sudden decrease in light intensity the growth rate is reduced or arrested because their energy metabolism becomes light limited. (Chory and Kaplan, 1983) The reduction in light intensity however initiates immediately an increased synthesis of pigment-protein complexes. Thus, light is not only a substrate for energy metabolism but also a signal which triggers a process of cell differentiation. After a

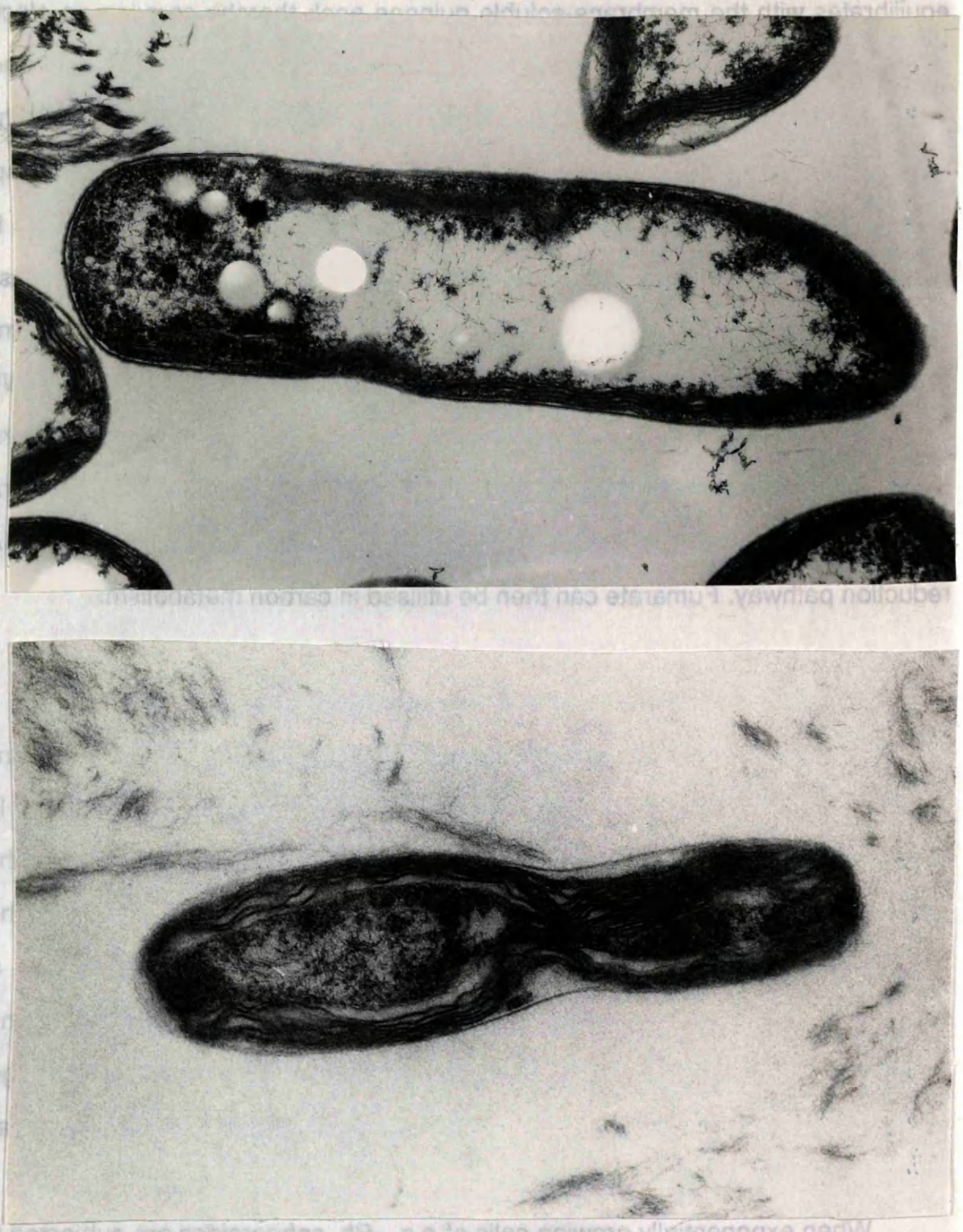


**Figure 1:4:** Electron micrographs showing the increased amount of ICM when *Rps. acidophila* 7050 is grown at high (approx. 160µmols/s/m²) [top] or low (approx. 10µmols/s/m²) [bottom] light intensity. Although an increased PSU size allows the cell to harvest more of the available photons, it is the increase in the surface area of the ICM, apparent here as an increase in the lamellae number, which accounts for the majority of the increased light-harvesting capability.

few hours, cells are adapted to low light intensity and continue to grow at the same rate as before the light intensity was lowered (unless the reduction in light intensity was too drastic), and the cells are no longer energy-limited.

In cells adapted to low light the amount of ICM is greater relative to cells grown under high light intensity, illustrated in Figure 1.4 for *Rps. acidophila* strain 7050. The number of photosynthetic units (PSU), measured as the number of RC per cell, increases and the size i.e., cross-sectional area, of the PSU, given as the number of antenna Bchl molecules, also increases. Thus, the light harvesting potential of the cell is increased. Typical data for *Rb. sphaeroides* is given in Table 1.1, reproduced from Kiley and Kaplan, (1988).

**Table 1.1 Adaptation of *Rb. sphaeroides* to decreasing light intensity.**

| Light Intensity ($W/m^2$) | Generation Time (h) | Specific Bchl. (µg/mg of cell protein) | ICM vesicles (per µm cell membrane) |
|---|---|---|---|
| 100 | 3.0 | 2.9 | 6.47±1.9 |
| 10 | 3.0 | 4.6 | 11.10±2.5 |
| 3 | 10.8 | 9.1 | 21.5±4.2 |

Light intensity is also known to affect the composition of the PSU as well as its cross-sectional area in *Rps. acidophila, Rps. palustris, Rps. cryptolactis* and in a member of the Chromatiaceae, *Chromatium vinosum*, however this latter species will not be mentioned further.

With *Rps. acidophila* three wild type isolates are commonly used, these are 7750, 7050 and 10050. When cultured at high light intensities all three produce a peripheral antenna complex that shows an absorption spectrum in the NIR that is typical of B800-850 i.e., similar to *Rb. sphaeroides,* they have Bchl a : carotenoid ratios of 2:1 and contain rhodopin and its glucoside as their main carotenoids. These B800-850 complexes have been called Type I and are characterised by the

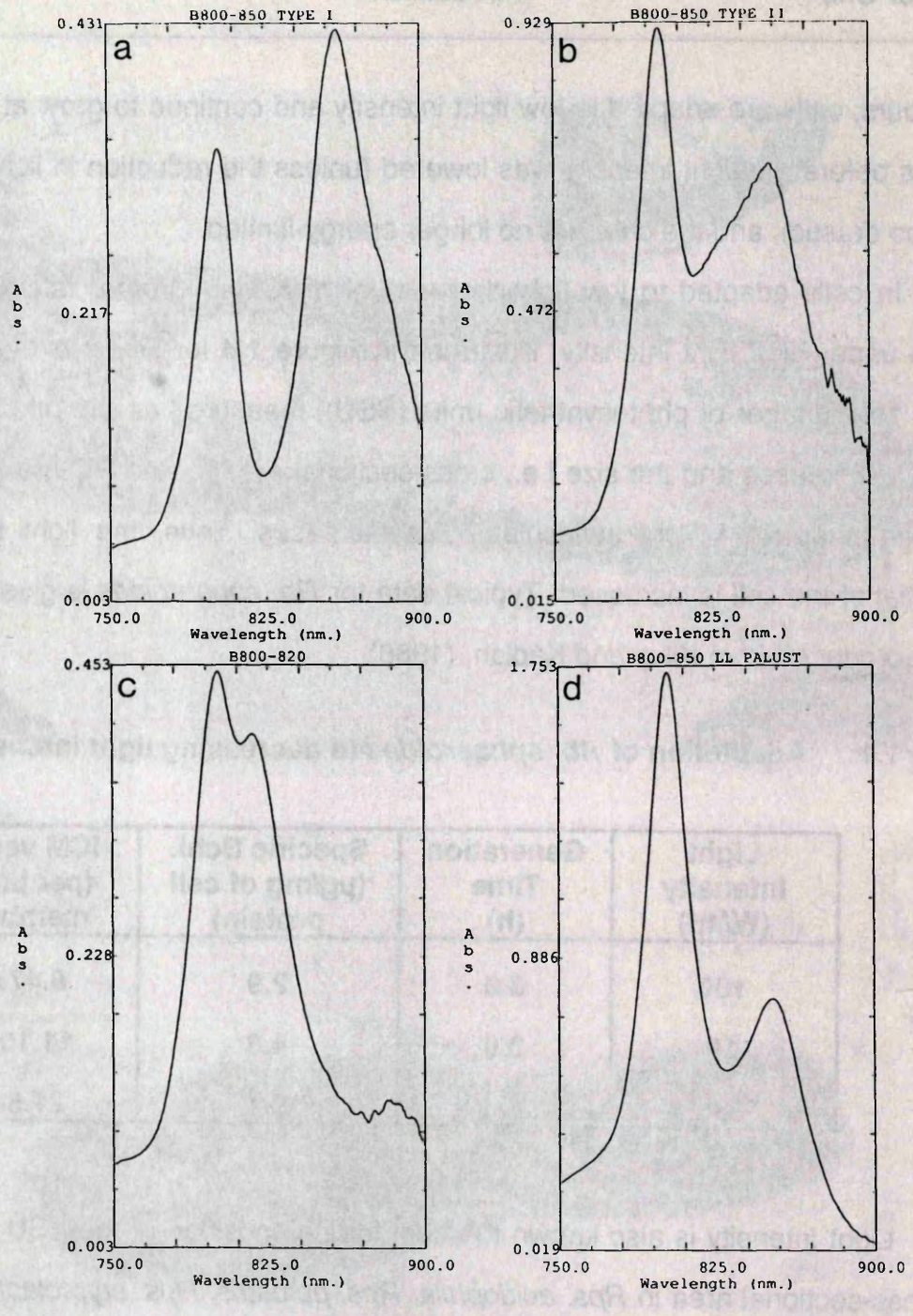



**Figure 1.5:** *Rps. acidophila* 7050 NIR absorption spectra; **(a)** Type I B800-850, **(b)** Type II B800-850, **(c)** B800-820 and the **(d)** *Rps. palustris* 2.1.6 low light B800-850 complex.

850nm absorption band being approx. 1.5 times as intense as the one at 800nm (Figure 1.5a). When *Rps. acidophila* 7050 is grown at reduced light intensities the B800-850 complex absorption spectra is altered in that the intensity of the 850nm band is equal to or lower than that of the 800nm band. This type of B800-850 complex is called Type II (Figure 1.5b). It was also observed that whenever a bacterium contained Type II B800-850 complex it also seemed to produce a different sort of peripheral antenna called B800-820, in which the red absorption maximum is shifted from 850nm to 820nm (Figure 1.5c) [Thornber *et al.,* 1978]. The B800-820 complexes from different *Rps. acidophila* strains are variable in their molecular regulation, Bchl a : carotenoid ratios and carotenoid composition. (Heinemeyer and Schmidt, 1983; Evans, 1989). The results presented in this thesis will elucidate further the relationship between these variables for both B800-850 and B800-820 complexes to the extent that it will be asserted that the division into Type I and Type II B800-850 complexes is now obsolete.

*Rps. palustris* also varies the composition of its peripheral antenna according to the light intensity at which it is grown (Hayashi *et al.,* 1982; Evans *et al.,*1990). The B800-850 complex from differently cultured cells varied greatly in their NIR absorption characteristics i.e., high light cells synthesise a *Rb. sphaeroides* B800-850 like complex, whereas low light induces a high 800nm and a low 850nm band, Figure 1.5d, using biochemical methods, the two spectrally different peripheral antennae of *Rps. palustris* have been shown to vary in (a) their pigment composition, the Bchl a : carotenoid ratios for high and low light are 2:1 and 3:1 respectively (Evans, 1989), and (b) their polypeptide composition.

## 1.5 Carotenoid functions and biosynthesis in the Rhodospirillaceae.

Carotenoids are a class of unsaturated hydrocarbons and their oxygenated derivatives which consist of eight isoprenoid units. By nature they are hydrophobic molecules which when associated with photosynthetic membranes are non-

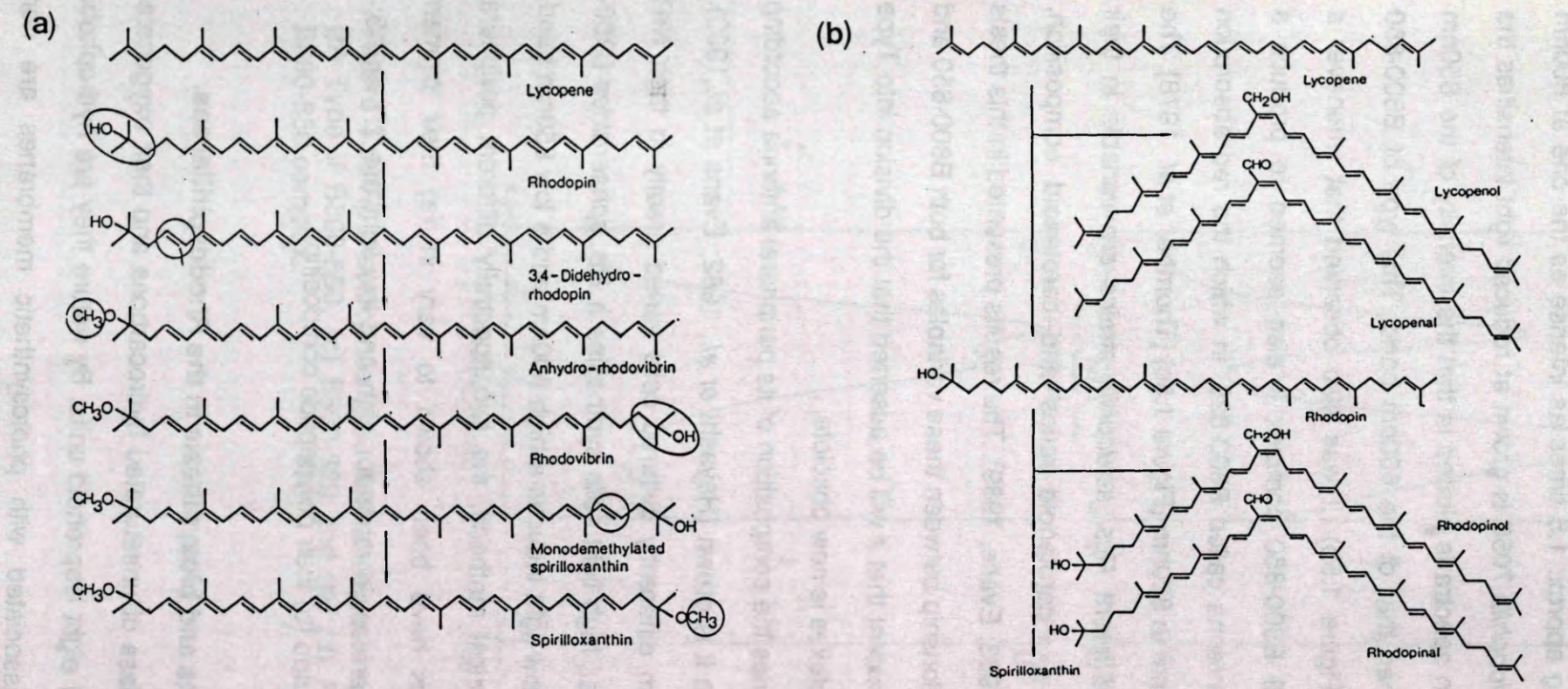



**Figure 1.6: (a)** Production of spirilloxanthin from lycopene via the 'normal spirilloxanthin pathway'. The new function introduced at each step is circled. **(b)** Branch pathway of the 'normal spirilloxanthin pathway' forming cross-conjugated carotenals. This is utilised by some members of the species *Rps. acidophila* under certain environmental conditions.

covalently bound to specific pigment-protein complexes, rather than freely mobile within the lipid bilayer.

Carotenoids have been shown to perform two major functions with regard to photosynthesis (Cogdell and Frank, 1987): (1) photoprotection, by quenching Bchl (or chlorophyll) triplet states before they can interact with molecular oxygen, thereby preventing singlet oxygen production. In addition the possibility also exists to quench this species should it be formed, however, this is not necessary *in vivo* as quenching of Bchl triplet states is exceedingly efficient and (2) light-harvesting. Although the former purpose is undoubtedly the most important, it is the light-harvesting role which is of interest in this study. Carotenoids as light-harvesting pigments absorb light-energy in the 450-570nm region and transfer it to the main light-harvesting pigment, Bchl. By performing this function, carotenoids extend the useful range over which photons are able to be harnessed for driving photosynthesis.

In the biosynthesis of carotenoids which are important in Rhodospirillaceae photosynthesis, the first $C_{40}$-intermediate formed is colourless phytoene, which is then desaturated to lycopene via phytofluene and neurosporene. Lycopene is the starting carotenoid for the 'normal spirilloxanthin series' which is the most important carotenoid biosynthetic pathway in the Rhodospirillaceae (and also for many members of the Chromatiaceae) and results in the formation of spirilloxanthin. This pathway is outlined in Figure 1.6a.

This pathway was elucidated from studies with *R. rubrum* S1, this bacterium accumulates up to 91% of its total carotenoid as spirilloxanthin (Schmidt, 1978). Other species accumulate intermediates of the pathway rather than the end product, e.g., *Rps. palustris* 2.1.6 with 73% as rhodovibrin and only 7% as spirilloxanthin. A pertinent example in the context of this work is *Rps. acidophila* which accumulates rhodopin rather than spirilloxanthin in all strains. Furthermore, the occurrence of an unusually high content of carotenoid glucosides (i.e., the sugar moiety forms a glycosidic linkage with the carotenoid hydroxyl group) was shown to

be characteristic of this species (Schmidt, 1978). Moreover, some strains of this species are able to utilise a branch of the pathway, illustrated in Figure 1.6b, that converts rhodopin to rhodopinal when grown under low light conditions (Heinemeyer and Schmidt, 1983). A different pathway for the formation of spirilloxanthin originating from neurosporene is also possible and is used by *Rb. sphaeroides* which accumulates one of the intermediates, sphaeroidene.

## 1.6 **Photosynthetic antenna systems.**

The absorption range of antenna systems of the diverse photosynthetic organisms runs from ≈360nm (Bchl Soret band) to 1000nm (Bchl). There is a broad, but not absolute, distinction between the spectral range of oxygenic photosynthetic organisms (300-700nm, in cyanobacteria, algae and plants) and anoxygenic photosynthetic organisms (700-1000nm, in bacteria). Thus through the evolutionary process it is not surprising that many types of antenna system have evolved in the various diverse photosynthetic organisms. As result of adaptation to varied and selective environmental conditions, photosynthetic organisms show a multitude of antenna structures of which four main types can be differentiated.

(1) Hydrophobic transmembrane polypeptides in intramembrane antennae of bacteria (Rhodospirillaceae, Chromatiaceae, Chlorobiaceae and Chloroflexaceae).

(2) Polypeptides with transmembrane and globular domains which comprise the antenna complexes of algae and higher plants.

(3) Globular, hydrophilic antenna polypeptides in extramembrane systems of Cryptophyceae, red algae and phycobilisomes in cyanobacteria (phycobiliproteins).

(4) Polypeptides in extramembrane antennae of chlorosomes in green photosynthetic bacteria (Cholorobiaceae and Chloroflexaceae).

### 1.6.1 Structural and functional principles of light harvesting antennae.

Most data on the structure and function of integral membrane antenna complexes i.e., on the specific interactions between carotenoid and chlorophyll molecules and polypeptides, has been obtained, non-coincidentally, on the simplest system which is that of the photosynthetic bacteria. The following principles, therefore, are mainly applicable to this group, and in particular the Rhodospirillaceae.

Fundamental to the formation of a functionally active antenna system, one in which energy transfer is directed from the antenna to the reaction centre with high efficiency, is the structure and organisation of the antenna polypeptides. The role of these polypeptides with respect to the hierarchical organisation of the whole antenna can be described as follows:

(1) All pigment molecules, with the exception of the bilin chromophores, are bound specifically, although non-covalently to polypeptides and form defined antenna complexes.

(2) Polypeptides form a scaffold which is responsible for maintaining the position, distance, orientation and environment of the pigment molecules. A possible exception is in the chlorosomes where there is a great deal of contention as to whether or not such protein is actually present.

(3) Polypeptides have specific association characteristics in forming oligomeric and multimeric units. This is the basis of the three-dimensional structure of the antenna and of the specific arrangement of the pigments.

(4) The antenna polypeptides associate in a hierarchical order, allowing the formation of antenna complexes with a functional grouping of pigments on an individual level, through to formation of the entire antenna by association of antenna complexes.

(5) This hierarchy of polypeptide association is the basis of the hierarchy of antenna complexes with varying absorption maxima, the formation of specific core complexes close to the reaction centre, and of peripheral antenna

complexes around the B890/RC conjugate. Therefore, as energy flows from short- to long-wavelength absorbing complexes, a system for heterogeneous (i.e., directed) energy transfer to the reaction centre is set-up. In this way and by spatial separation of pigment clusters in the antenna complexes, random walk is relatively minimised.

### 1.6.2 Correlation of conserved amino acids with antenna absorption characteristics.

This efficient directional flow of excitation energy to the reaction centre is possible by a combination of chemically identical pigments absorbing at different wavelengths (Zuber, 1986a). Thus, the absorption characteristics of the pigments are determined mainly by their specific microenvironment (pigment-pigment and pigment-protein interactions); Bchl when in solution (acetone: methanol, 7:2) has its absorption maxima at approx. 772nm, however upon binding to their specific antenna polypeptides a large bathochromic shift into NIR and hyperchromism of the positive $Q_y$ band is induced.

Since 1981, when for the first time the primary structure of an intramembrane-bound antenna polypeptide became available (Brunisholz *et al.*, 1981), a large number of equivalent light-harvesting polypeptides from other strains of purple bacteria have been sequenced. Alignments of these polypeptide primary sequences have revealed strongly conserved amino acid residues which were presumed, often verified since by experimental investigation, to be of major importance in the functioning of the antenna.

As previously intimated, individual species of Rhodospirillaceae vary in the extent of their heterogeneous energy transfer systems. *R. rubrum* (B890) and *Rps. viridis* (B1020) have only the core complex, *Rb. sphaeroides* and *Rb. capsulatus* have two (B875, B800-850), whereas *Rps. acidophila* and *Rps. cryptolactis* are able to produce, depending on the growth conditions, three different complexes (B890, B800-850 and B800-820). The basic structural and functional element of Rhodospirillaceae antenna complexes, both core and the peripheral antenna, is a

heterodimer of small α- and β-polypeptides, each consisting of 50-60 amino acid residues and an $M_r$ of about 5-7kD. In three species so far known a third non-pigmented gamma polypeptide is also present: they are the B1015 complex of *Rps. viridis* (approx. 4kD) [Brunisholz *et al.*, 1985], the analogous complex in *Et. halochloris* (approx. 3kD) [Zuber and Brunisholz, 1991], and the B800-850 complex of *Rb. capsulatus* (approx. 14kD) [Tichy *et al.*, 1991]. However definitive evidence on the precise function of these polypeptides within their respective complexes is as yet lacking.

When prediction algorithms were used (Argos *et al.*, 1982) to detect transmembrane spanning helices and averaged over the aligned polypeptides, a single central hydrophobic core region was indicated for α- and β-core and peripheral polypeptides separating two hydrophilic domains. The predicted location of the C- and N- terminal domains is on the surface of, or in the polar head region of the membrane. Cleavage of part of the N-terminal domains of the α- and β-polypeptides on the cytoplasmic side of the membrane by partial hydrolysis with proteolytic enzymes demonstrated their orientation in the membrane (Brunisholz *et al.*, 1986). These results fixed the localisation of the N- and C-termini of α- and β-polypeptides on opposite sides of the membrane as only one helix traverses the lipid bilayer, a conclusion subsequently confirmed by UV-CD measurements (Cogdell and Scheer, 1985), moreover the transmembrane helices of the antenna complex were proposed to be tilted to the plane of the bilayer (Breton and Nabedryk, 1984). Aligned primary sequences of the core and peripheral polypeptides are given in Figure 1.7a (α-polypeptides) and 1.7b (β-polypeptides).

An overall comparison of the sequences of antenna polypeptides shows low homology (7-13%) between α- and β-subunits indicating an early separation of their genes during the course of evolution. The homology between α-polypeptides of the B890/875 and B800-850/B800-820 is higher (13-28%), the same being true for β-polypeptides, however, the homology for equivalent polypeptides in different species can be as high as 78%.

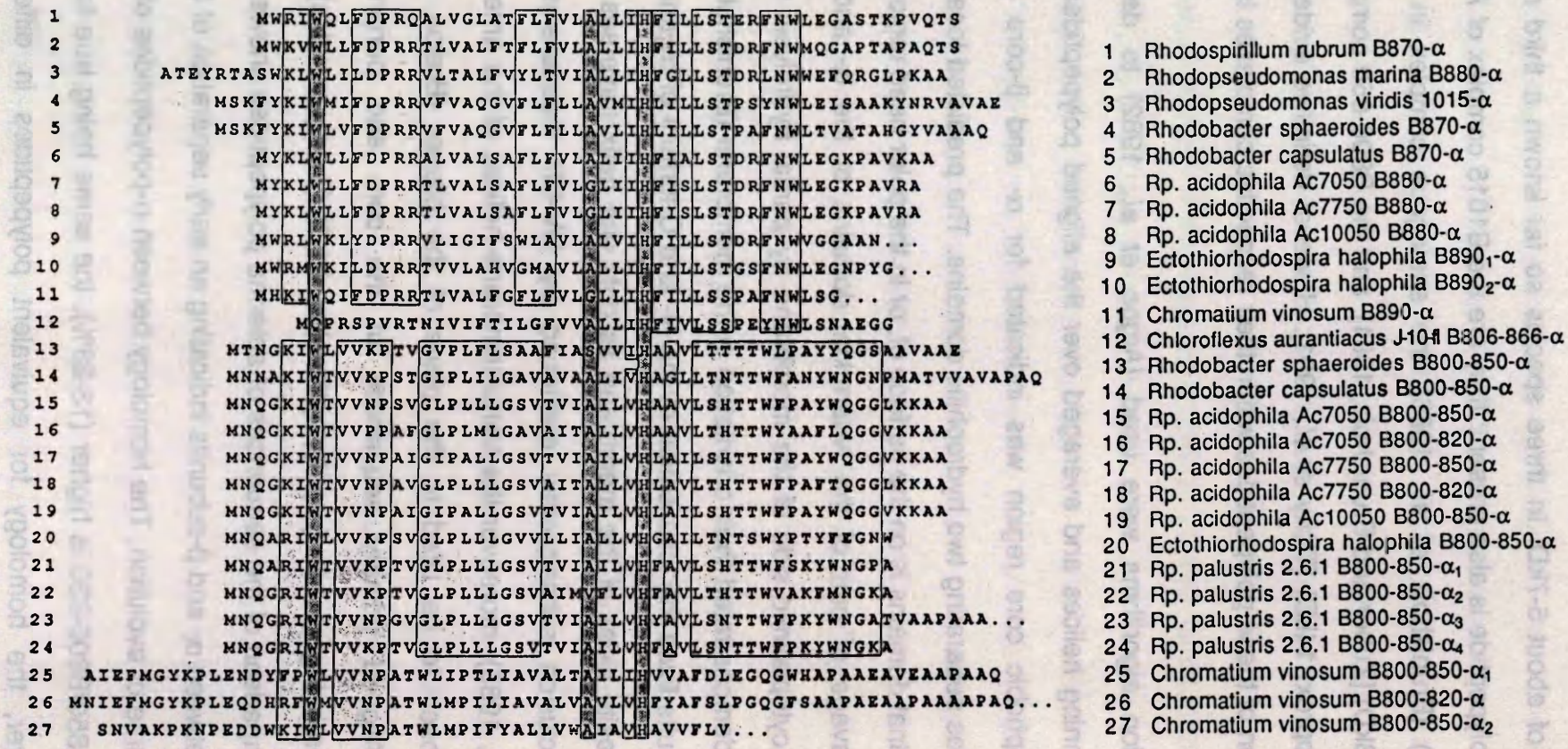
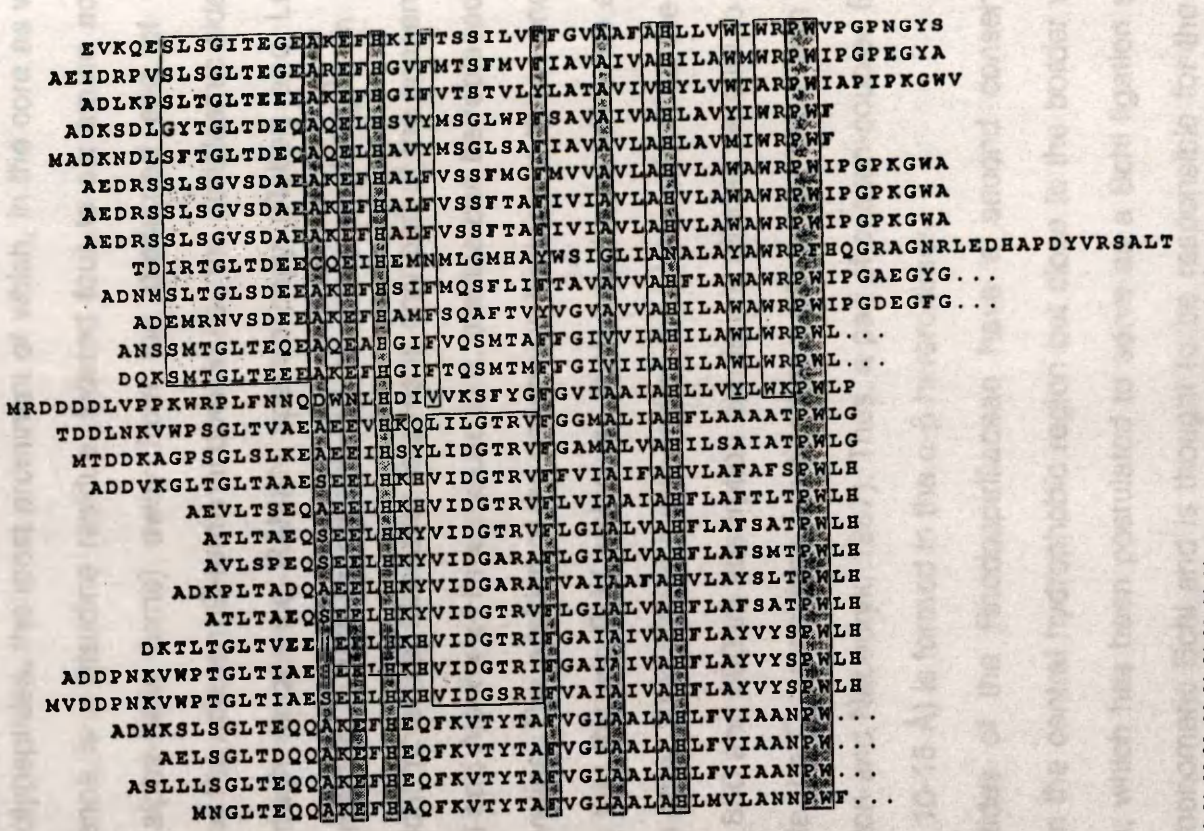



**Figure 1.7a:** Primary structures of the α-polypeptides from the core (B875/B890, B1015) and peripheral antenna complexes of purple bacteria. The boxes denote putative conserved structure elements, of which the most important is the Bchl binding A-x-x-x-H- motif. (Sequences taken from Zuber, 1990).



1 Rhodospirillum rubrum B890-β
2 Rhodopseudomonas marina B880-β
3 Rhodopseudomonas viridis B1015-β
4 Rhodobacter sphaeroides B870-β
5 Rhodobacter capsulatus B870-β
6 Rp. acidophila Ac7050 B890-β
7 Rp. acidophila Ac7750 B890-β
8 Rp. acidophila Ac10050 B890-β
9 Ectothiorhodospira halochloris β-Polypeptid
10 Ectothiorhodospira halophila $B890_1$-β
11 Ectothiorhodospira halophila $B890_2$-β
12 Chromatium vinosum $B890_1$-β
13 Chromatium vinosum $B890_2$-β
14 Chloroflexus aurantiacus J-10-fl B806-866-β
15 Rhodobacter sphaeroides B800-850-β
16 Rhodobacter capsulata B800-850-β
17 Rp. acidophila Ac7050 B800-850-β
18 Rp. acidophila Ac7050 B800-820-β
19 Rp. acidophila Ac7750 B800-850-β
20 Rp. acidophila Ac7750 B800-820-$\beta_2$
21 Rp. acidophila Ac7750 B800-820-$\beta_1$
22 Rp. acidophila Ac10050 B800-850-β
23 Rp. palustris 2.6.1 B800-850-$\beta_1$
24 Rp. palustris 2.6.1 B800-850-$\beta_2$
25 Rp. palustris 2.6.1 B800-850-$\beta_3$
26 Chromatium vinosum B800-850-β
27 Chromatium vinosum B800-820-$\beta_1$
28 Chromatium vinosum B800-820-$\beta_2$
29 Chromatium vinosum B800-820-$\beta_3$

**Figure 1.7b:** Primary structures of the β-polypeptides from the core (B875/B890, B1015) and peripheral antenna complexes of purple bacteria. The boxes denote putative conserved structure elements. With these polypeptides there are two potential Bchl binding sites (Sequences taken from Zuber, 1990).

There are conserved amino acid residues in the hydophobic central domain of both the α- and β-polypeptides, the most prominent of which, in the core as well as the peripheral antenna is a histidine residue, located four to six amino acids (equal to 1 to 1½ alpha-helix turns) away from the presumed end of the hydrophobic stretch and the adjacent C-terminal domain i.e., the periplasmic side. It is now accepted, through resonance RAMAN spectroscopy (Robert and Lutz, 1985), that this His residue is fifth-ligand bonded to the central $Mg^{2+}$ in the porphyrin ring of the Bchl molecule. Moreover this His is only part of the conserved motif Ala-x-x-x-His in β and Ala-x-x-x-His-x-x-x-Leu in α, alanine can be replaced by residues smaller than valine. The position of the conserved histidine in the α- and β-polypeptides suggests that the two Bchl molecules ligated to the histidine are at a close distance to each other. This idea is supported by the observation that the $Q_y$ transitions of both the 850 and 890nm absorption bands are aligned parallel to the membrane plane, while the $Q_x$ transitions are tilted at a 55° angle away from the membrane plane (Breton and Nabedryk, 1987). Thus, a pair of exciton-coupled Bchl molecules (separation 10-15 Å) is formed in the α-β-heterodimer.

The β-polypeptides of the Rhodospirillaceae have a second conserved histidine, located within the central hydrophobic region but close to the border with the N-terminal domain, which has been postulated to serve as a Bchl ligation site. This is the so-called monomeric Bchl and is thought to be responsible for the $Q_y$ transition absorbing at 800nm in the B800-850 complex. However, it is noticeable that the B890 complex which has an analogous N-terminal His residue does not bind Bchl, and resonance RAMAN spectroscopy which confirmed the C-terminal His as the fifth ligand to the 850nm absorbing Bchl $Mg^{2+}$ in the B800-850 complex has been unable to do this for the N-terminal His and the 800nm Bchl.

A number of other conserved amino acid residues have been highlighted which are probably of structural or functional significance, illustrated in Figure 1.7. These residues often comprise a grouping of 2-4 amino acids, of which most contain 1-2 aromatic residues. Studies with other chromophore binding polypeptides

like heme-binding proteins, rhodopsins, bacteriorhodopsin and the Bchl a binding protein of *Prosthecochloris aestuarii* has led Brunisholz *et al.*, (1988) to propose that, as a general principle aromatic residues in the vicinity of the chromophore play a significant role in the specific spectral properties of the particular chromoprotein, possibly through interaction of the delocalised electron clouds. Evidence for this 'aromatic residue spectral shift' was obtained when the structure of the *Rps. viridis* reaction centre was determined to 2.8Å resolution, the special pair of the reaction centre interacts with eleven aromatic residues (Michel *et al.*, 1986). More tenuously, the gamma polypeptide already briefly mentioned in the extremely far-red shifted antennae of Bchl b containing species, is enriched in aromatic amino acids (Brunisholz *et al.*, 1985).

One aromatic amino acid-antenna polypeptide interaction is of particular importance with regard to this study. Brunisholz and Zuber, (1988), after comparing the peripheral α-polypeptides from *Rps. acidophila* have proposed that a mutation has occurred in the C-terminal domain that has changed the aromatic cluster from $Y_{44}$-$W_{45}$ in the B800-850 α-apoproteins to FL (strain 7050) and FT (strain 7750) in the B800-820 α-apoproteins. The precise nature of the interaction between the aromatic residues and the Bchl-dimer awaits tertiary structure determination; the obvious candidate being between the π-electron clouds of the aromatic ring and that of the porphyrin ring; however, (Robert, B., pers. comm.) has recently proposed from resonance RAMAN spectroscopy with *Rb. sphaeroides* ($Y_{44}$-$Y_{45}$) that a H-bond between the 2-acetyl group of the Bchl molecule and the hydroxyl group of one of the tyrosine residues is also important. This H-bond contributes to the situation in which the Bchl-dimer absorbs at 850nm, when the amino acid sequence is FL or FT this H-bond is broken and the absorpbance maxima is blueshifted to 820nm.

The interaction between the aromatic residues and the Bchl-dimer was tested experimentally using site-directed mutagenesis by Fowler *et al.*, (1992), who suggested that there is a close spatial relationship between tyrosine residues on the

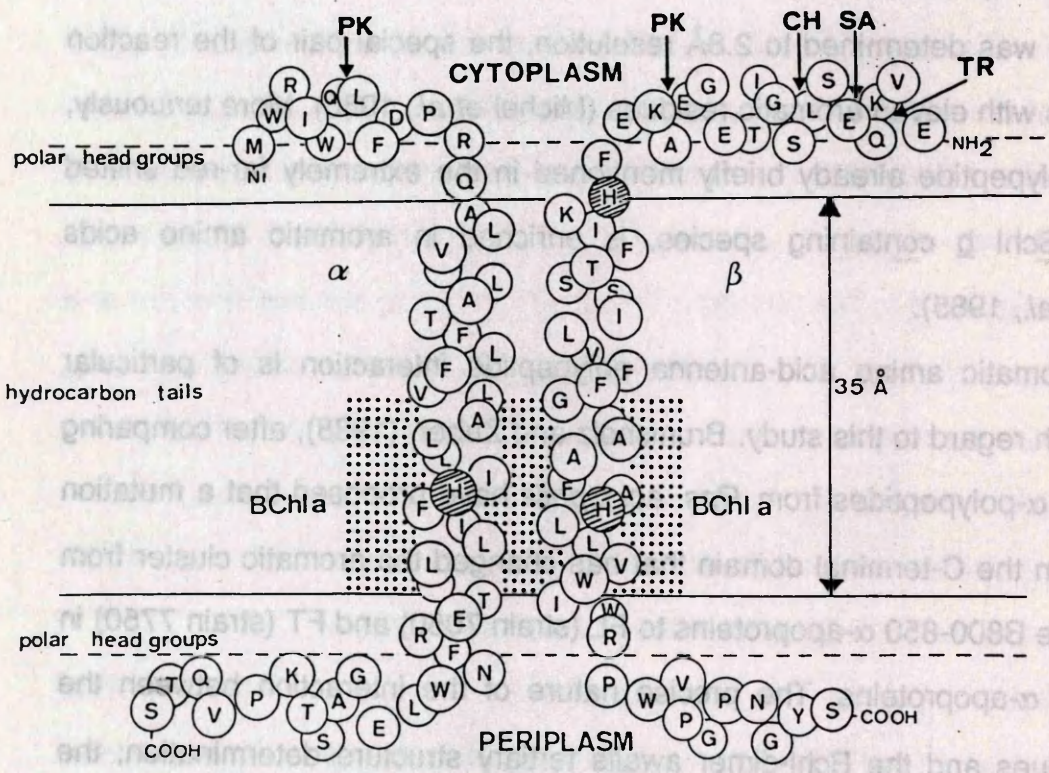



**Figure 1.8:** Schematic diagram of the transmembrane orientation of the α-β-heterodimer from the *R. rubrum* B890 complex, with respect to the photosynthetic membrane, as revealed by proteolytic digestion of the accessible surface oriented polypeptide domains. (Reproduced from Zuber, 1986a). PK, proteinase K; CH, chemotrypsin; SA, *Staphylococcus aureus* proteinase; TR, trypsin.

α-polypeptide and Bchl in the *Rb. sphaeroides* B800-850 complex, and that this proximity may promote redshifts *in vivo*. A clear correlation was observed between the absorbance of Bchl and the presence of two tyrosine residues, $Y_{44}$-$Y_{45}$ on the α-subunit. By constructing single ($Y_{44}$-$Y_{45}$→FY) and double ($Y_{44}$-$Y_{45}$→FL) site-specific mutants, the absorbance of Bchl was blueshifted by 11 and 24nm respectively at 77K.

Thus, it is apparent that upon pigment binding not only will gross bathochromic shifts occur but that the pigment can also be subjected to a degree of 'fine tuning' brought about by residues in its vicinity: in this case the Bchl dimer responsible for the $Q_y$ transition absorbing at 850nm is induced into absorbing at 820nm, with all the concomitant implications therein for the light harvesting capability of the cell. Combining the results above from all observations and experiments has enabled Zuber, (1986b) to postulate a tentative model of how the α-β-heterodimer is arranged with regard to the ICM bilayer, presented schematically in Figure 1.8.

### 1.6.3 Three dimensional peripheral antenna complex structure.

Figure 1.8 is the starting point for any consideration of the quaternary structure of antenna complexes. It represents the minimum structural-functional folding unit although it has no independent or separate existence, rather this α-β-heterodimer-helix pair with its tri-partite domain structure is the starting point for specific interactions which lead to the association of these repeating units into larger aggregates and eventually into the mature complex. Zuber, (1990) asserts that it is interactions between polar residues of the alpha helix on the cytoplasmic side of the membrane which are the prime forces responsible for the aggregation of the polypeptides, and that tilting of the alpha-helices results in optimum packing via the side-chains rather than interactions between the polar N- and C-terminal domains. However there is no experimental evidence for either possibility.

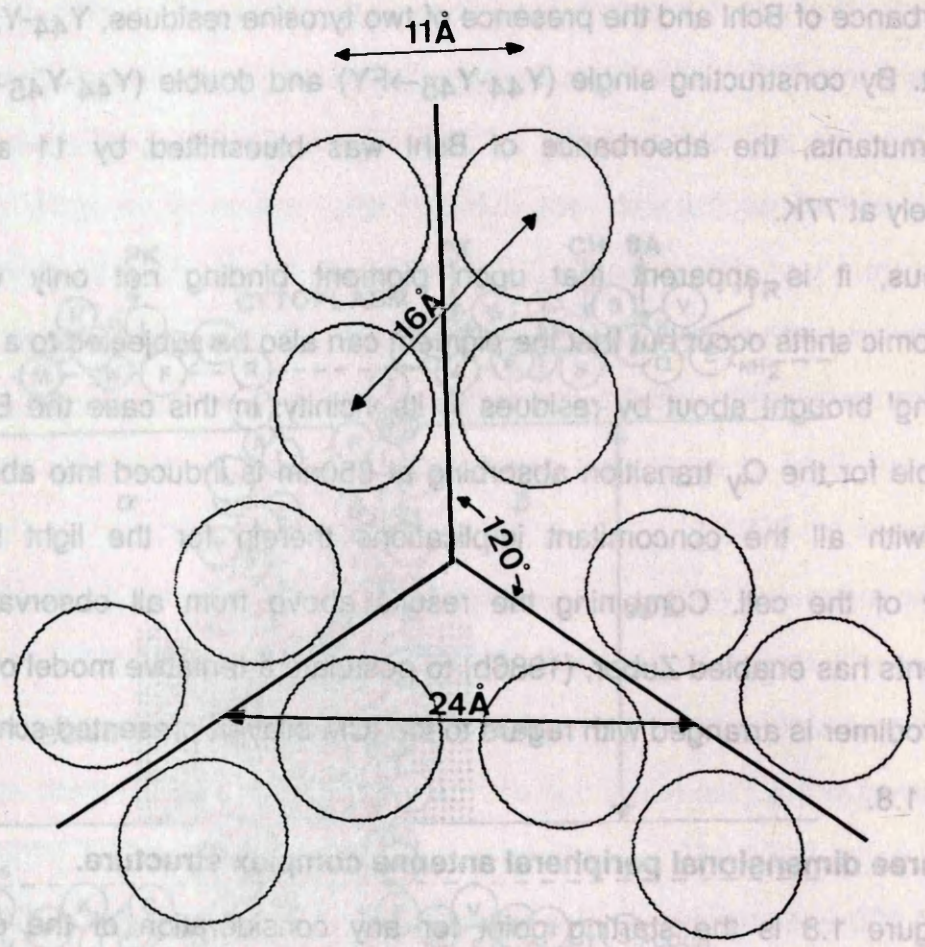



**Figure 1.9:** Tentative model for the spatial orientation of the alpha helices in the B800-850 complex from *Rps. acidophila* 10050. This arrangement has been obtained from a Paterson projection electron density map. Each circle represents an α-helix viewed down its longitudinal axis.

The smallest intact, pigmented, native peripheral antenna complex that is formed appears to be a hexamer of the α-β-heterodimer-helix pair i.e., 12 polypeptides with 18 Bchl and 9 carotenoid molecules. This is supported primarily by X-ray crystallographic data (Cogdell, R. J., pers. comm.), theoretical modelling (Zuber, 1990) and biochemical evidence (Papiz *et al.,* 1989; Braun and Scherz 1991).

Nuclear Magnetic Resonance and Electron diffraction are extremely useful techniques when applied to small molecules, however, X-ray crystallography is the only method available which ultimately leads to complete structural data for proteins at high atomic resolution. Reaction centre complexes from both *Rps. viridis* and *Rb. sphaeroides* have been crystallised. The structure of RCs from *Rps. viridis* has been determined to a resolution of approx. 2.3Å (Deisenhofer and Michel,1989), whereas that of *Rb. sphaeroides* has been determined to 2.8Å (Allen *et al.,* 1986). Recently, an improved data set leading to higher resolution (2.1Å) for this latter species has been reported. (Michel, H. pers. comm.). A resolved structure of light-harvesting antenna complexes from Rhodospirillaceae has proved elusive; crystals have been obtained that refract to 3.2Å from *Rps. acidophila* (Cogdell, R. J., unpublished results) and to 2.4Å from *R. molischianum* (Michel, 1991), however the phase problem as yet precludes attainment of the structure. By utilising the electron density map by means of a Patterson projection, inferences can be made as to the number and spacial separation of alpha helices. The current data for the B800-850 complex of *Rps. acidophila* 10050 is presented as a tentative model in Figure 1.9. It can be seen that the α-β-heterodimer-helix pair has combined to form a dimeric unit i.e., 4 polypeptides, that is repeated three times about an axis rotated 120° to form the complete antenna complex. This structure is consistent with the data obtained so far for the B800-820 complex from a different *Rps. acidophila* strain, 7750 (Guthrie *et al.,* 1992). There is no suggestion, however, from these results that the α-helices are tilted to the membrane normal. This dimeric unit would appear to be analogous to the 'minimal unit' previously proposed (Kramer *et al.,* 1984) for the

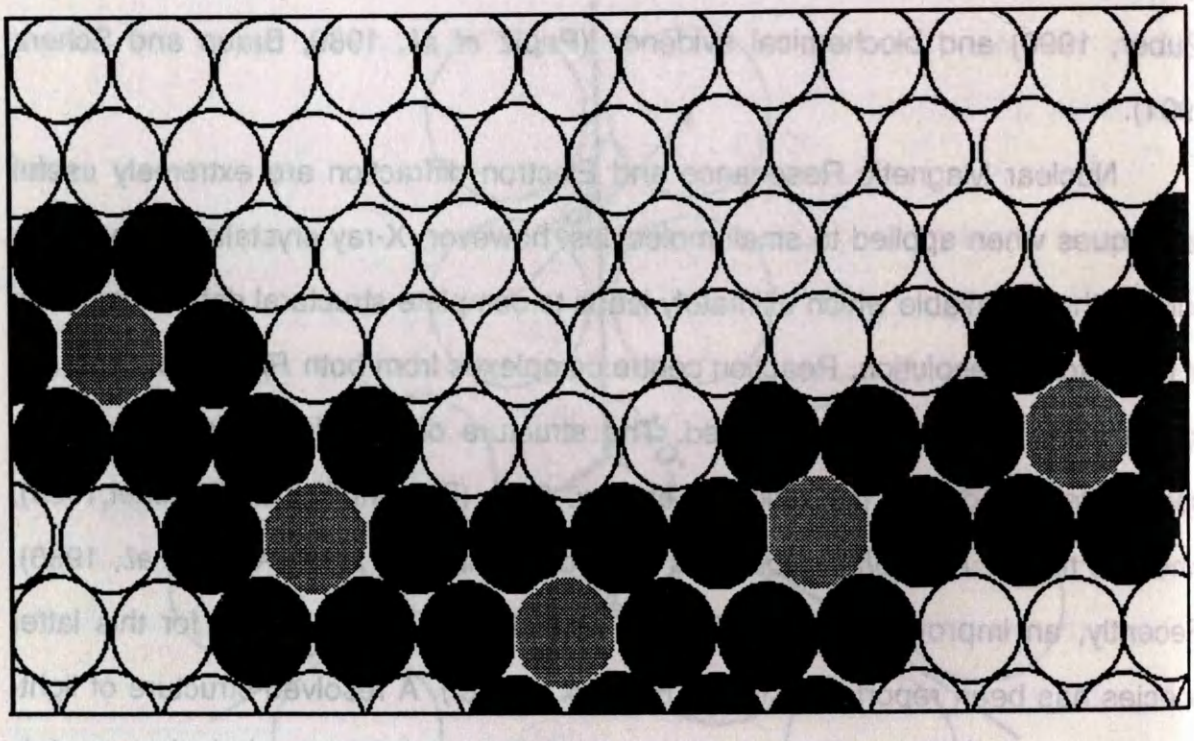


**Figure 1.10:** Monger and Parson, (1977) 'lake' model for the arrangement of the photosynthetic apparatus within the ICM for *Rb. sphaeroides.* This model allows migration of excitons from one B875/RC conjugate to another because B875 complexes surround RCs but also form channels interconnecting them. Open circles, B800-850; Closed circles, B875; Grey circles, RC.

B800-850 complex from *Rb. sphaeroides* which also consists of an $\alpha_2$-$\beta_2$ arrangement. Thus, in the model the $Q_x$ Bchl transitions, of the 850nm absorbing pair at least, are aligned perpendicular to the plane of the membrane and the $Q_y$ transition aligned in the plane thereby facilitating exciton transfer. The Bchl molecules in the B800-850 complex of *Rps. acidophila* 10050 are presumed to be consistent with this model.

Ultimately, X-ray crystallography yields exact data on the structure of antenna complexes i.e., the minimal repeated unit that can be isolated intact, however it cannot reveal the precise arrangement of antenna complexes within the PSU. Unfortunately, there is much conjecture and little hard evidence in the literature as to the topographic organisation / interrelationship of reaction centres, B890 complexes in the form of cyclic dodecamers and B800-850 (B800-820) cyclic hexamer complexes, within the ICM. Perhaps the most useful model as a conceptual starting point was forwarded by Monger and Parson, (1977). This has become known as the 'lake' model, Figure 1.10, and was conceived from fluorescence studies that demonstrated that excitation energy within a B800-850 complex can be delivered to any one of several reaction centres. The PSU therefore consists of several reaction centre cores surrounded by shared B800-850 antenna, resulting in an increase in the efficiency of light harvesting due to the ability of an exciton to migrate away from a reaction centre that is oxidised, i.e., 'closed' to one that has just been reduced i.e., 'open'.

## 1.7 Exciton transfer via antenna complex pigments.

Consider a molecule e.g., Bchl that has two electronic (energy) levels, the lower or ground state ($S_0$) and one upper or first excited state ($S_1$) and some of the vibrational levels of each. Upon absorption of a photon its energy is used to promote an electron from $S_0$ to $S_1$; provided the selection rules defined by quantum mechanics are adhered to. Any excess energy will be absorbed as vibrational energy and so the electron resides in one of the vibrational energy levels. This

vibrational energy is rapidly dissipated as heat and the electron drops to the lowest vibrational level of $S_1$; if the electron remains at this level for a relatively long time the probability increases that it will decay to $S_0$ by emitting light, a process called fluorescence. Because energy is lost in dropping to the lowest level of $S_1$, the emitted light will have less energy (i.e., longer wavelength) than the absorbed light. However, in returning to $S_0$ the electron may arrive at one of the vibrational levels of $S_0$ instead of the absolute ground level, and this vibrational energy will be dissipated as heat. Hence if there are many absorption events, the light emitted will have many wavelengths (all longer than that of the absorbed light); the probability of dropping from the first excited state to each vibrational level of the ground state determines the shape of the fluorescence spectrum.

Furthermore, the transition from $S_1$ to $S_0$ is possible through processes other than fluorescence i.e., (1) by energy transfer to a neighbouring pigment molecule, (2) by intersystem crossing to give the triplet state, (3) by non-radiative heat loss and (4) by photochemistry. These processes compete with each other and the proportion of energy dissipated by each is mechanism is dictated by the chemistry and geometry of the pigments.

At any instant the intensity of the fluorescence emitted from a particular pigment type within the PSU indicates the proportion of excited molecules in that pigment type (Clayton, 1980). This is because each excited molecule has a certain probability per unit time of emitting a quantum of fluorescence.

### 1.7.1 Simple algebraic description of fluorescence.

The concept of fluorescence and the respective competing decay processes can be described mathematically. Consider a population of N pigment molecules that have been excited; their rate of decay is given as:

$$\frac{dN}{dt} = -(k_f + k_{nr} + k_{et} + k_{other})\, N \qquad (1)$$

where, $k_f$, $k_{nr}$, $k_{et}$ and $k_{other}$ are the rate constants (probabilities) of decay by fluorescence, non-radiative heat loss, energy transfer to another molecule and photochemistry respectively. The actual observed lifetime of an excited population is denoted by:

$$\tau = \frac{1}{(k_f + k_{nr} + k_{et} + k_{other})} \quad (2)$$

A lifetime is the time taken for a population of N molecules to decay to N/e excited molecules, where e is the base number of natural logarithms. A high rate constant for one of the pathways of decay means that the probability of this pathway being chosen is high compared to the probabilities of the competing pathways.

The quantum yield of fluorescence i.e., the proportion of molecules decaying by the fluorescence pathway is given by:

$$\Phi_f = \frac{k_f}{(k_f + k_{nr} + k_{et} + k_{other})} \quad (3)$$

Similarly the energy transfer efficiency is denoted by:

$$\Phi_{et} = \frac{k_{et}}{(k_f + k_{nr} + k_{et} + k_{other})} \quad (4)$$

If fluorescence emission is the only route available for decay then the lifetime, known as the intrinsic fluorescence lifetime can be given by:

$$\tau_f = \frac{1}{k_f} \quad (5)$$

Combining equations (2), (3) and (5) a linear relationship is obtained which describes how the actual, and the fluorescence lifetimes are proportional to the fluorescence yield:

$$\emptyset_f = \frac{\tau}{\tau_f} \qquad (6)$$

By measuring the fluorescence at different wavelengths which correspond to the different pigment types, the distribution of excitation can be determined either in steady state under constant illumination, or on a transient timescale using picosecond flash photolysis. Time-resolved studies can be used to investigate the rates of each of the above competing processes and by implication, provide information about the mechanisms of energy transfer.

Upon absorption of a photon by an antenna pigment the exciton is transferred from one molecule to another by a more or less random walk and reaches the reaction centre within $10^{-11}$-$10^{-10}$s. Two fundamentally different types of energy transfer are relevant to antenna systems: energy transfer according to the Förster mechanism (Förster, 1948) and the Dexter electron exchange mechanism (Dexter, 1953).

### 1.7.2 **Förster mechanism.**

This energy transfer mechanism occurs by inductive resonance and was first described by Förster, (1948); it does not involve the exchange of electrons, rather is based on an weak, electric dipole-dipole interaction. The rate of energy transfer between a donor (D) and an acceptor (A) can be expressed as:

$$k_{DA} = \frac{1}{\tau_f}\left(\frac{R_O}{R_{DA}}\right)^6 \qquad (7)$$

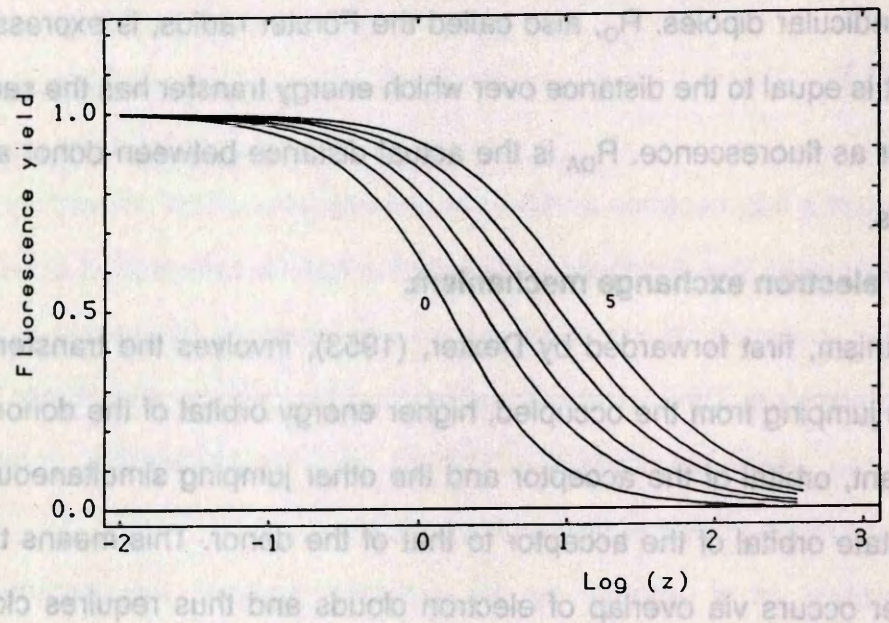
in which $\tau_f$ is the lifetime of an exciton if decay of the excited state would only occur via fluorescence. $(R_O)^6$ is a function of the overlap integral between the emission band of the excited, donor molecule and the absorption band of the acceptor molecule, and of their mutual orientation i.e., maximal for parallel dipoles and zero for mutually perpendicular dipoles. $R_O$, also called the Förster radius, is expressed in units of length; it is equal to the distance over which energy transfer has the same probability to occur as fluorescence. $R_{DA}$ is the actual distance between donor and acceptor molecules.

### 1.7.3 **The Dexter electron exchange mechanism.**

This mechanism, first forwarded by Dexter, (1953), involves the transfer of two electrons, one jumping from the occupied, higher energy orbital of the donor to the same but vacant, orbital of the acceptor and the other jumping simultaneously from the ground state orbital of the acceptor to that of the donor. This means that the energy transfer occurs via overlap of electron clouds and thus requires close contact between the donor and acceptor ($\leq$ 5Å), and is the mechanism of transfer usually invoked to account for the light-harvesting role of carotenoids.

### 1.7.4 **Singlet-singlet annihilation and domain sizes.**

When intense laser flashes of sub-ns duration are used for performing fluorescence measurements of fluorescence yield, or the lifetimes of the excitons in the antenna systems, the results depend on the laser flash intensity used. The fluorescence yield as well as the lifetime will be reduced when the intensity is increased. This is due to collision between multiple excitons within the same antenna. The precise mechanism of the interaction is not clear, although it can be envisioned by describing the process in terms of a Förster-like energy transfer from a Bchl in its first excited singlet state ($S_1$) to a neighbouring Bchl which is also in the $S_1$ state. This results in a Bchl in the ground state and one in a higher excited state ($S_n$). This latter state, however, has a generally very short lifetime ($<10^{-13}$s) and will decay rapidly to $S_1$ without emitting fluorescence. The net result is then the loss of one exciton that is transformed into heat. This process,




Paillotin master equation:

$$\phi(z) = \phi(0) \cdot r \sum_{k=0}^{\infty} \frac{(-z)^k}{r(r+1)(r+2)\cdots\cdots(r+k)} \frac{1}{k+1}$$

where Ø(0) is the low intensity fluorescence yield observed with all RC 'closed', and *z* is a dimensionless variable proportional to the laser pulse intensity.

**Figure 1.11:** Plot of the Paillotin master equation, illustrating calculated values of the fluorescence quantum yield (Ø) as a function of *z*, for increasing values of the parameter r. In general, the curves differ from each other in shape and in position with respect to the abscissa. As the value of r increases, the curves drop off less steeply and are shifted to the right on the horizontal log (*z*) scale.

$$S_1 + S_1 \rightarrow S_0 + S_n \rightarrow S_0 + S_1 + \text{heat}$$

is called singlet-singlet annihilation. It does not occur (or at least very rarely) under natural photosynthetic conditions because the density of excitons is too small.

The probability for two excitons to 'collide' depends not only on the exciton density but also on the rate of exciton migration over the antenna and on the size of the array of Bchl molecules among which energy transfer can occur. Therefore, studies of singlet-singlet annihilation provide an insight into these parameters.

Information about the number of connected antenna Bchls, called the domain size (Clayton, 1967), and about the rate of exciton migration within the antenna (Vos *et al.,* 1986) can be deduced using a plot of the fluorescence yield versus pulse intensity by means of equations derived by Paillotin *et al.,* (1979), Figure 1.11. They introduced a master equation approach to describe the shape of the resulting annihilation curve, and introduce the annihilation parameter r:

$$r = \frac{2(\text{Averaged rate of mono-exciton decay})}{\text{Rate of annihilation per pair of excitons.}} \qquad (8)$$

It can be seen from Figure 1.11 that not only the slope of the curve depends on the value of r but at high values of r the curves are shifted to the right, i.e., greater exciton densities are needed to produce the same amount of annihilation as for r = 0. This can be explained as follows, r = 0 corresponds to the situation when the amount of mono-exciton decay is very much less than the amount of annihilation, which will be the case in small domains with efficient energy transfer. Under such circumstances two excitons present in one domain will be certain to annihilate. When in contrast mono-exciton decay and annihilation are of the same magnitude, there is a certain probability that one of the two excitons will be lost before they are able to find each other because of the increased domain size This

results in a decreased efficiency of annihilation, therefore higher exciton densities are needed to give the same amount of annihilation as for small values of r.

Thus in the experimental set-up, comparison of a measured annihilation curve with Figure 1.11 gives the value of r and the size of the domain. Since the mono-exciton decay rate is usually known it is possible to calculate mathematically rates for light-harvesting and transfer, provided that the excitation distribution is always random, implying that the domain is structurally homogeneous. Thus measurement of annihilation curves can provide important information about the sizes of the domains for energy transfer and the rate constants involved. Such information can be very useful in understanding the organisation of complex photosynthetic antenna systems.

## 1.8 Regulation of peripheral antenna genes.

To date the two species of bacteria which have received the most attention from molecular geneticists are *Rb. sphaeroides* and *Rb. capsulatus*; as a result many of the key genes encoding the photosynthetic apparatus in these two species have been identified, cloned and sequenced. The structural genes for two of the RC polypeptides, RC-L and RC-M, designated pufL and pufM respectively, are transcriptionally linked to the two structural genes for the alpha and beta subunits of the B875 complex, pufA and pufB respectively (Zhu *et al.*, 1986). The structural gene for the third polypeptide in the RC, RC-H (designated puhA), is not linked to the puf operon, and data from *Rb. capsulatus* suggest it maps at least 40kb away (Taylor *et al.*,1983).

The structural genes for the B800-850 $\alpha$ and $\beta$ subunits are also linked to one another and comprise the puc operon.

### 1.8.1 Organisation of the puc operon.

The genes encoding the B800-850 $\alpha$- and B800-850 $\beta$-apoproteins, the puc operon, have been identified, cloned and sequenced by designing oligonucleotide primers from the known polypeptide sequences, in *Rb. capsulatus* (Youvan and

Ismail, 1985), *Rb. sphaeroides* strain 8253 (Ashby *et al.,* 1987) and strain 2.4.1 (Kiley and Kaplan, 1987). The following features are common in all three cases: the gene for the β-polypeptide precedes that for the α-polypeptide, only 14-20 bases separate the two genes, each gene is preceded by a putative Shine-Dalgarno ribosome binding site, and there is no cleavable signal sequence preceding the mature polypeptide. Interestingly, the start codon for pucB in *Rb. sphaeroides* strain 2.4.1 is GTG.

Further work has established that sequences downstream from pucBA are essential for formation of the B800-850 complex in both species. However, this research has also uncovered differences in puc operon organisation in this region between *Rb. sphaeroides* and *Rb. capsulatus,* the main one being the identification of the gene coding for the 14kb unpigmented γ polypeptide associated with the B800-850 complex of *Rb. capsulatus*. a comparable polypeptide is not present in *Rb. sphaeroides.*

Tichy *et al.*, (1989) sequenced 3kb downstream from pucBA in *Rb. capsulatus* and found three open reading frames (ORF) of 403, 113 and 118 codons, since designated pucC, pucD and pucE respectively. Amino acid sequence studies suggest that pucE is the gene for the gamma polypeptide mentioned above. Previously (Kaufmann *et al.*, 1884), this group had used transposon mutagenesis to produce mutants that were photosynthetically defective although the organisation of the genes was at this time unknown; one such mutant, NK3, was shown to be B800-850$^-$. Tichy *et al.*, (1989) showed that this mutant's phenotype was due to Tn5 insertion in pucC indicating that this gene product is necessary for B800-850 formation. Further work (Tichy *et al.,* 1991) successfully managed to restore the B800-850$^+$ phenotype in NK3 by placing pucCDE under the control of the nifHDK promoter and growing the cells under limiting nitrogen; pucCDE on their own were not able to complement NK3 indicating that there is no transcription initiation site between the end of pucA and pucC. From this, these authors defined the puc

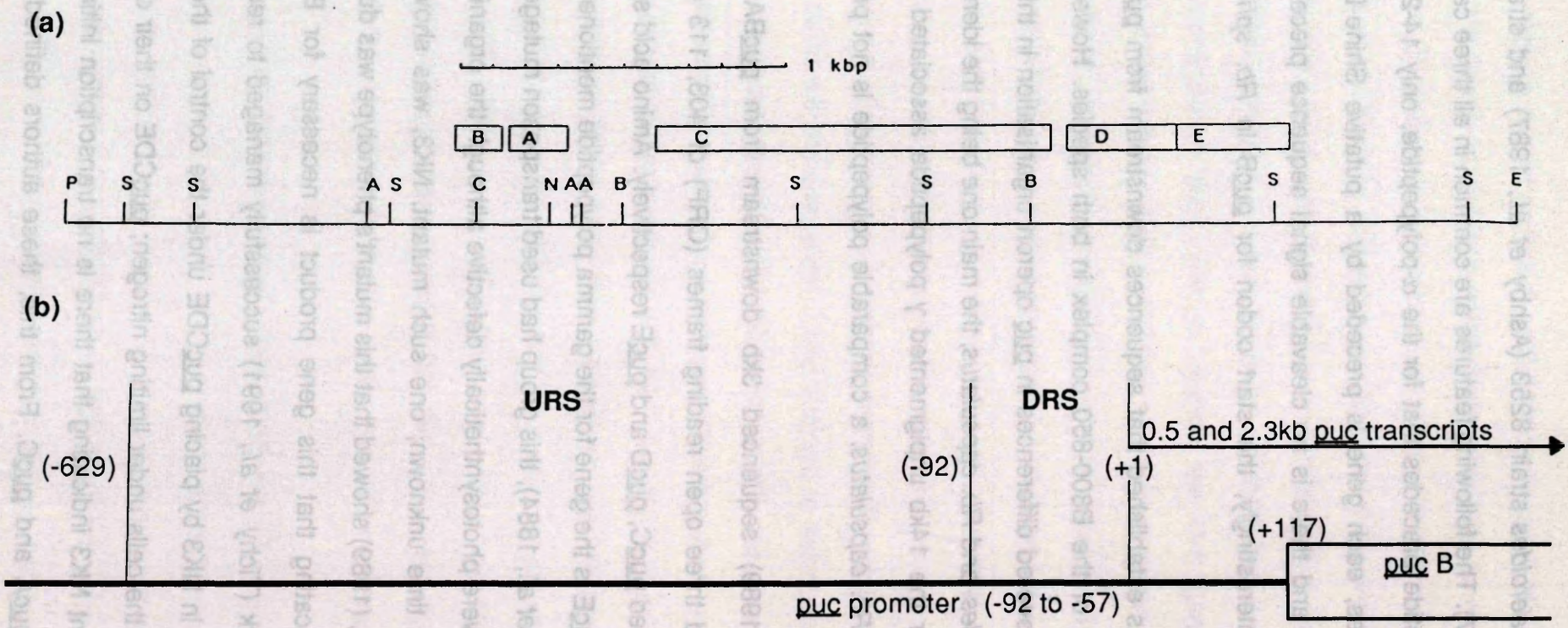



**Figure 1.12: (a)** Representation of the pucBACDE operon in *Rb. capsulatus*. Genetic and physical map of the 4.7kb EcoRI-PstI fragment containing the genes and ORFs for pucB, A, C, D and E. (A, ApaI; B, BalI; C, ClaI; E, EcoRI; N, NruI; P, PstI; S, SmaI). **(b)** Upstream regulatory elements involved in the transcription of the puc operon in *Rb. sphaeroides.* The DRS appears to be associated with anaerobic control and URS with oxygen control. (Other more speculative control regions have been omitted). Compiled from Lee and Kaplan, 1992a and b.

operon in *Rb. capsulatus* as containing pucBACDE, and is represented schematically in Figure 1.12a.

With regard to the *Rb. sphaeroides* puc operon; the emphasis has been placed on the transcription of the genes and identifying regions upstream of pucBA which are responsible for the control of the operon by oxygen and light, rather than characterising ORFs downstream of pucBA. That in fact this downstream region (1.8kb) is necessary for B800-850 formation, was demonstrated by Lee *et al.*, (1989), who also identified two pucBA-specific transcripts which have 5' ends at 117bp upstream of the start of pucB. Moreover Lee and Kaplan, (1992a) have begun to dissect *cis*-acting regulatory elements upstream of this transcription startpoint in an attempt to begin to understand the role of oxygen and light in puc operon expression. These authors have delineated two functionally separable *cis*-acting domains: the upstream regulatory region (URS) (-629 to -92) is responsible for enhanced transcriptional regulation of puc operon expression by oxygen and light. The more proximal upstream region (downstream regulatory region [DRS]), containing putative promoter(s) and other regulatory sequences (-92 to -1), is involved in unenhanced transcriptional expression of the puc operon under aerobic and anaerobic conditions. By utilising puc::lacZ transcriptional fusions containing serial 5' or internal deletions of DNA sequences upstream of pucBA, Kiley and Kaplan propose that the DRS shows normal derepression of puc operon expression when cells are shifted from aerobic to photosynthetic growth conditions in terms of percentage change but does not show the potential range of expression that is only observed when elements of the URS are present. They have also introduced a distinction between anaerobic control (describing the shift) and oxygen control (describing the magnitude of derepression). The URS itself can be regarded as having two functional domains, the proximal domain appears to be associated with some form of light activation and the distal domain with oxygen repression of the puc operon and that these two regions have some form of hierarchical relationship with oxygen repression predominant; the upstream region of the puc operon in *Rb.*

*sphaeroides* is shown schematically in Figure 1.12b. In an accompanying paper Lee and Kaplan, (1992b), have attempted to try and identify *trans*-acting factors involved in puc operon regulation, which are able to effect both URS and DRS. However, this particular aspect of these authors' work is presently the subject of much controversy.

In summary, these studies suggest that the organisation and regulation of the puc operon and its expression is exceedingly complex, revealing the presence of numerous *cis*-acting upstream regulatory elements and several linked *trans*-acting elements, as well as downstream elements/ORFs and possibly, additional *trans*-acting elements mapping over 1000kb away from the puc operon.

A further degree of complexity appears to be present in *Rps. palustris* strain 1e5, because Tadros and Waterkamp, (1989) have reported that there are multiple copies of the coding regions for the B800-850 complex apoproteins. As previously mentioned this species contains more apoproteins in its B800-850 complex than either *Rb. sphaeroides* or *Rb. capsulatus,* of which $\alpha_a$ and $\alpha_b$ along with $\beta_a$ and $\beta_b$ comprise the major polypeptides. They used an oligonucleotide derived from the amino terminal end of the $\alpha_a$ polypeptide to screen a genomic library and were able to show that this species contains at least four different clusters of beta and alpha genes. Two of these clusters corresponded the $\beta_a/\alpha_a$ and $\beta_b/\beta_a$ polypeptides, the two remaining were termed $\beta_c/\alpha_c$ and $\beta_d/\alpha_d$ because they did not correspond to any previously purified polypeptides, although the authors suggest that all four are expressed. However, these four clusters were characterised from four different clones, therefore they are likely to be spatially separated and as such not contained within a single operon, which will in turn raise interesting questions as to their co-ordinate regulation.

### 1.8.2 Post-transcriptional and post-translational puc regulation.

Lee *et al.,* (1989), showed that in *Rb. sphaeroides* there are two puc specific transcripts, 0.5 and 2.3kb, which both have 5' ends mapping to 117 nucleotides upstream of the start of pucB. The smaller transcript is approx. 200-fold more

abundant than the larger transcript and encodes the pucBA structural genes. Previously Kiley and Kaplan, (1987), had demonstrated that the cellular level of the 0.5kb transcript was both oxygen and light regulated; barely detectable levels of puc-specific transcript were observed in aerobic cells. This is in contrast to the results obtained with the genes encoding the other Bchl-encoding proteins (e.g., puf and puh), whose mRNAs are relatively abundant in chemoheterotrophically grown cells (Donohue *et al.*,1986). Under photohetertrophic conditions the level of this small transcript increases three- and four-fold at 10 and 3 $W/m^2$ respectively, compared with growth at 100 $W/m^2$.

Lee *et al.*, (1989), also suggested that the 0.5kb pucBA-specific transcript in an B800-850$^-$ mutant (PUC-Pv), which was constructed by insertional interruption of the DNA downstream of pucBA, is translated but that one or both of the translated polypeptides are turned over very rapidly due to their instability caused by the absence of gene product(s) encoded by the puc downstream region. The accumulation in the downstream mutant strain of pigments (presumably Bchl precursors) offers the possibility, long suspected, that products in porphyrin ring biosynthesis are required for B800-850 complex synthesis. In addition it has been shown in *Rb. capsulatus* (Dierstein, 1983) that the pigment-binding polypeptides appear to be labile in the absence of Bchl, suggesting that the presence of Bchl (or some precursor) is essential for the stabilisation of these polypeptides in the ICM. These authors suggest, therefore, that the region downstream of pucBA may encode gene products involved in pigment processing or the coupling of pigment synthesis to expression of B800-850 spectral complex formation, which then implies that the level of the 0.5kb puc-specific transcript is not the sole factor in determining the cellular level of B800-850 in the ICM. Rather, post-transcriptional control mechanisms, such as translation of the pucBA transcript, stability of the B800-850 alpha and beta polypeptides and assembly into the mature complex, are most likely also important factors which modulate the ultimate cellular level of the B800-850 complex.

Zucconi and Beatty, (1988), have also proposed a similar mechanism of puc regulation in *Rb. capsulatus.* When *Rb. capsulatus* were grown photosynthetically, four times as much B800-850 mRNA was found in cells grown with high light as in cells grown with low light, yet low light cells contained four times as many B800-850 complexes. Therefore, as the amounts of mature pucBA gene products found in cells grown with high or low light intensity do not bear a direct relationship to the level of transcription, this suggested that the amount of complexes present in the ICM are the result of post-transcriptional (or post-translational) regulatory process. It has been shown (Dierstein, 1984) that the turnover rate of the B800-850 α-polypeptide in a Bchl-less mutant strain and in wild-type cells exposed to inhibitors of Bchl synthesis, is much greater than in untreated cells. This suggests that in the presence of Bchl, the B800-850 polypeptides may be protected from degradation, perhaps because of conformation change from Bchl-sensitive to Bchl-resistant after binding Bchl. High light cells have the capacity to produce four-fold more peptides than low light cells, due to higher levels of mRNA, however, since less Bchl is produced in high light grown *Rb. capsulatus,* more of the newly translated peptides would be turned over, as they would be unable to bind Bchl molecules. No free Bchl is detected in normal cells of photosynthetic bacteria, so the ratio of pigment-binding polypeptides to Bchl must be equal to or greater than one. The total Bchl content of anaerobically grown *Rb. capsulatus* increases as the light intensity decreases (Drews, 1986); therefore when cells are shifted from a high to low light environment, more Bchl is produced which would bind to the newly translated polypeptides; these protein-pigment complexes would then be more resistant to degradation. However, Zucconi and Beatty also note the possibility of control of translation of B800-850 mRNA, so that translation would be less efficient in cells grown at high light intensity although they make no attempt to distinguish between the post-translational and translational mechanisms.

Tichy *et al.,* (1991), demonstrated that the presence of the pucC gene product is necessary for high level expression of this operon in *Rb. capsulatus.* The

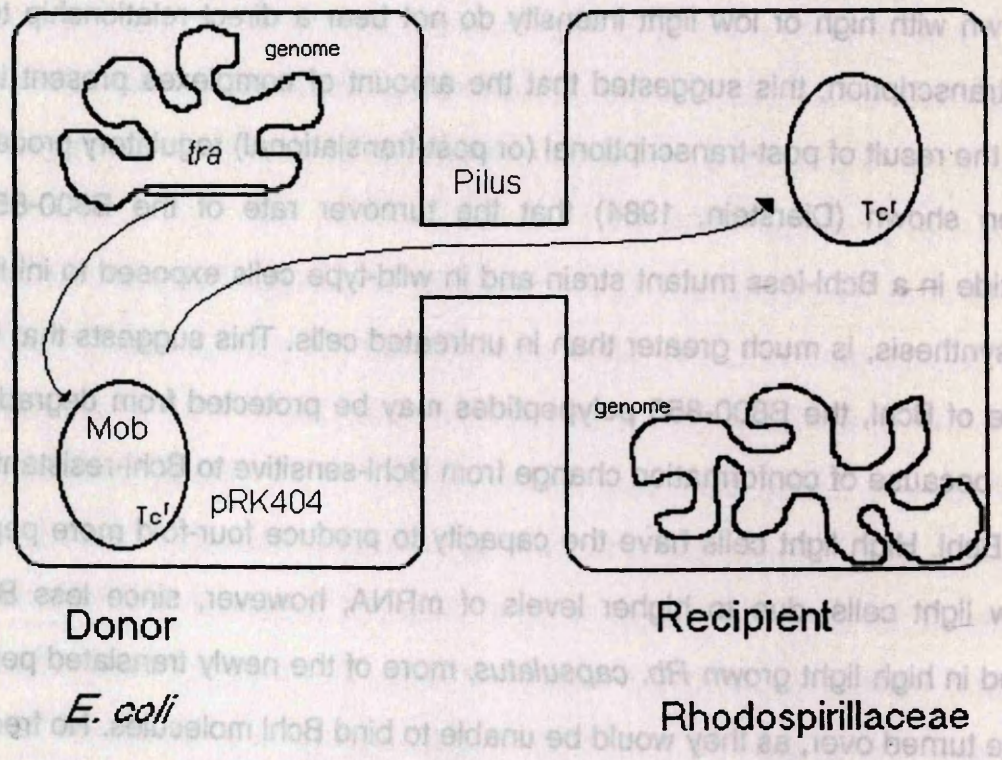



**Figure 1.13**: Schematic illustration of a di-parental mating, in this case the Tc^r conferring plasmid pRK404 is introduced into the recipient. Tra functions integrated on the donor chromosome effect mobilisation through the plasmid Mob site. The result is that the mobilisable vector is transferred into the recipient, conferring resistance to antibiotic i.e., tetracycline.

way in which this regulatory function of PucC is accomplished is not clear, however hydrophobicity analyses (Tichy *et al.*, 1989) classified PucC as an integral membrane protein which would suggest its influence on puc transcription is an indirect one, and that PucC is more involved in B800-850 assembly within the ICM. These authors further assert that the pucD gene product is also connected with B800-850 stabilisation.

## 1.9 Introduction of foreign DNA into Rhodospirillaceae.

The method of choice when introducing cloned DNA into *E. coli* is transformation, this routinely yields in excess of $10^7$ transformants per µg DNA. Transformation of a photosynthetic bacterium has only been reported by Fornari and Kaplan, (1982), for *Rb. sphaeroides*, however their procedure yielded only 1-5x$10^3$ transformants per µg DNA which decreased rapidly with increasing plasmid size. Moreover, transformation requires plasmids which lack sites for host restriction enzymes e.g., *Rb. sphaeroides* 2.4.1 contains RshI activity and it could be transformed by RSF1010 which lacks a RshI site. Cloning DNA which contained a RshI site into this plasmid prevented transformation.

Electroporation increases the yield by a factor of ten although the applicability of this technique is limited as the introduced DNA is still restricted. This drawback can be avoided by introducing plasmid DNA into the Rhodospirillaceae through bacterial conjugation.

### 1.9.1 Broad host range conjugal transfer.

Simon *et al.*, (1983), developed a di-parental mating system to introduce plasmid DNA into gram negative bacteria not closely related to *E. coli*; initially they used *Rhizobium meliloti*, however this method has since been shown to be equally applicable for *Rb. sphaeroides* (Hunter and Turner, 1988).

The system consists of two components: special *E. coli* donor strains (called mobilising strains), and derivatives of *E. coli* vectors which contain a specific recognition site for mobilisation (Mob site). The mobilising strains were constructed

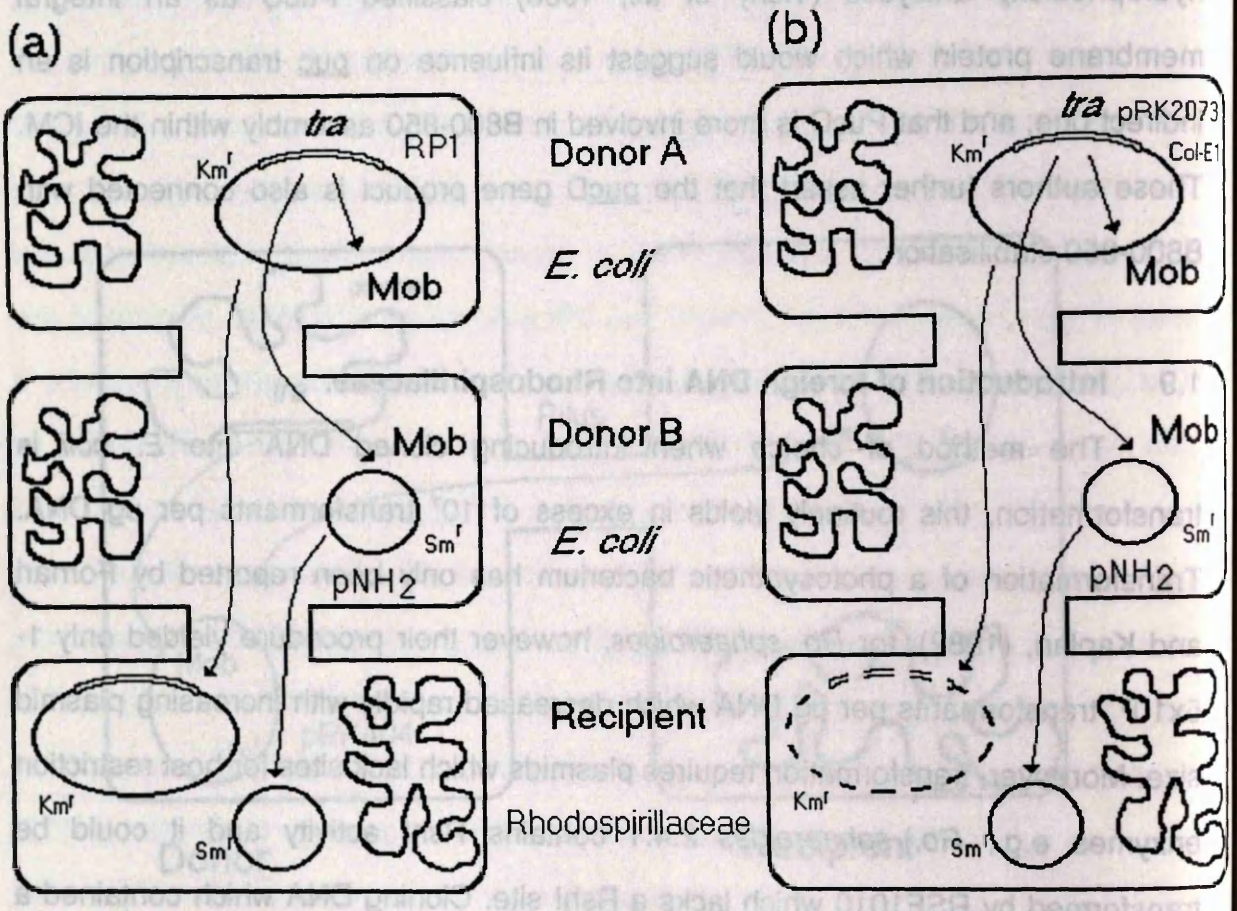



**Figure 1.14:** Schematic illustration of **(a)** a tri-parental mating that introduces two compatible plasmids into a recipient cell. In this case the tra functions from RP1 are used to mobilise itself and $pNH_2$ (the vector). As both plasmids are able to be maintained within the cell, the recipient will be resistant to both antibiotics $Km^r$ and $Sm^r$. **(b)** This mating also introduces two compatible plasmids, however, pRK2013 contains the ColE1 replicon in addition to the tra functions, as such it will be unable to replicate and so will be deleted .The result is that the recipient will be resistant to $Sm^r$ but susceptible to $Km^r$.

by integrating the broad host range plasmid RP1 (Thomas and Smith, 1987) into the chromosome of the donor, thus preventing self-transfer of this plasmid, and are now available to effect mobilisation. RP1 is a 60kb promiscuous plasmid which due to its large size is itself of no use as a cloning vector and the disparate nature of its genetic loci, until recently, precluded construction of smaller derivatives. A number of these loci are *trans*-acting factors, necessary for cell-cell contact, mating bridge formation, initiation and continuation of transfer replication in donor and recipient, and for overall control of these processes; these functions are termed collectively the transfer (tra) functions. The recognition site for these functions on the plasmid to be mobilised is called the Mob site and includes the origin of transfer (oriT). Selection of successful conjugation is accomplished because the plasmid also carries resistance markers, usually to antibiotics and, because the mobilising strains are auxotrophic, they are easily removed from recipient cells by plating on minimal media. A schematic illustration of a di-parental mating is shown in Figure 1.13.

Originally before the development of specially constructed mobilising strains, plasmids had to be mobilised tri-parentally. This involves two donor cells; one harbours a tra$^+$ plasmid and the other a Mob$^+$ plasmid, contingent upon both plasmids belonging to different incompatibility groups (plasmids that share either replication control or partitioning functions which compete for stable inheritance, are termed incompatible, and are placed in the same incompatibility group). The tra$^+$ functions are then able to effect mobilisation of the plasmid from which they were transcribed i.e., *cis*-acting and the other plasmid via the pilus i.e., *trans*-acting. This results in both plasmids residing in the recipient cell, a situation not always desirable in molecular cloning. The development, however, of self-transmissible helper plasmids that have only a limited replicative host range has negated this problem, due to the fact these plasmids contain the ColE1 replicon which is only functional in *E. coli*. When introduced into Rhodospirillaceae the plasmid is unable to replicate and is lost. The tri-parental system has retained a degree of applicability because it uses *E. coli* strains that are easily transformed, whereas mobilising

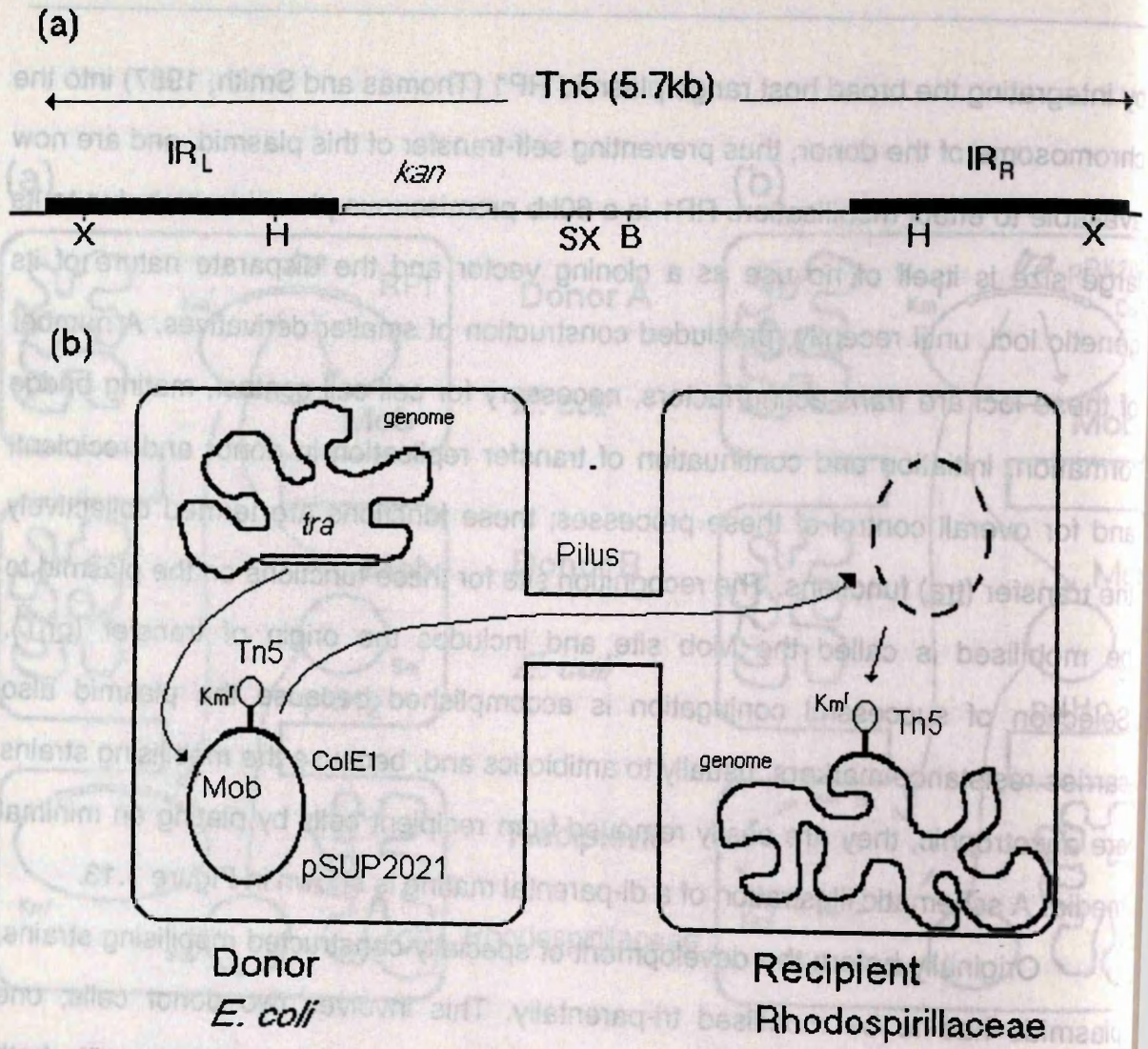



**Figure 1.15: (a)** Simplified genetic and physical map of Tn5. (IR, inverted repeat; *kan*, kanamycin 3'-phosphotransferase; H, HindIII, B, BamHI; S, SalI; X, XhoI). **(b)** Transposon mutagenesis of the Rhodospirillaceae genome. This di-parental mating introduces a ColE1 containing, 'suicide' vector harbouring a transposon (Tn5) into the recipient cell. The conjugal process is identical to that illustrated in Fig. 1.13. The transposition of Tn5 into the recipient's genome ensures this element will be carried into the next generation, because the plasmid is deleted from the cell. Hence, the antibiotic resistance of the recipient is conferred by the transposon and not by the vector.

strains tend to transform only with difficulty. Both types of tri-parental mating are shown schematically in Figure 1.14a and b.

### 1.9.2 **Transposon mutagenesis mediated via mobilising strains.**

Transposons are specific DNA segments with the ability to move as a unit in a more or less random fashion from one genetic locus to another (Berg and Berg, 1983); insertion of a transposon may occur within a gene, resulting in complete loss of gene function. Transposon Tn5 has been used most often for the apparently random construction of auxotrophs, or other mutants, the genetic/physical map is given in Figure 1.15a. In order to use a transposon as a mutagenic agent it must be loaded onto a plasmid that can be efficiently introduced into the organism to be mutagenised. Furthermore, a means for selecting against stabilisation of the transposon carrier in the recipient species is required. If these conditions are met, transposition events can be isolated simply by selecting for transposon-mediated antibiotic resistance.

Simon *et al.,* (1983), have developed the pSUP range of plasmids from pBR325 to fulfil these criteria. Transposons can easily be loaded onto a pSUP plasmid using a variation of the tri-parental mating procedure outlined in Figure 1.14, these plasmids are $Mob^+$ and are available with a variety of antibiotic markers which allow for maintenance of the antibiotic as well as the transposon. Finally they contain the ColE1 replicon and therefore act as 'suicide' donors. Once introduced into the recipient cell the plasmid is unable to replicate, as its replicon is non-functional, and will be deleted from the cell. Thus, this acts as strong selective pressure for the transposon to transpose into the genome of the recipient, and cells which have the transposon integrated into their genomes can be selected by plating on the antibiotic to which the transposon confers resistance. A schematic mating of this type is illustrated in Figure 1.15b.

# Chapter Two

# Materials and methods.

## 2.1 General culture storage.

Five species of Rhodospirillaceae were used in this study: *Rhodopseudomonas acidophila* strains 7050, 10050 and 7750, *Rhodopseudomonas palustris* strain 2.1.6, *Rhodobacter sphaeroides* strain 8253, *Rhodospirillum rubrum* strain S1 and *Rhodopseudomonas cryptolactis,* a recently isolated thermotolerant bacteria (Stadtwald-Demchick *et al.,* 1990). Cultures were maintained in agar stabs on the laboratory shelf until required, and checked to ensure purity on a regular basis. Additional stocks were prepared in 50% (v/v) glycerol and stored at -70°C.

All strains of *Escherichia coli* were maintained in 15% (v/v) glycerol at -70°C. Prior to the day when the cells were required, they were streaked out on LB-plates and incubated overnight at 37°C. The plate could be stored at 4°C for up to 2 weeks and colonies removed as required .The genotypes of all *E. coli* strains used in this study are listed under Appendix D.

## 2.2 Liquid cell culture.

### 2.2.1 Photoheterotrophic growth of Rhodospirillaceae.

*Rps. acidophila* was grown in Pfennigs' medium (Pfennig, 1969) with succinate as the carbon source. *Rps. palustris, Rb. sphaeroides* and *R. rubrum* were grown in a well defined media with succinate as the carbon source, whereas *Rps. cryptolactis* media utilised pyruvate. The composition of all media is given in Appendix A. For liquid cultures the appropriate stab was overlaid with media and placed in the growth room, illuminated at approximately 150µmol/s/m², at 30°C. After 1-4 days the cells were of sufficient density to be transferred to a 500ml flat-sided bottle, incubated for a further 24 hours then either harvested or further sub-cultured.

*Rps. acidophila* strains 10050, 7750 and 7050 were also grown at different light intensities and temperatures. For the light intensity experiments the cells were grown at increasing distances from an incandescent 40W bulb. Initially relatively close, to approximate growth room conditions i.e., high light. In each case the third

sub-culture was harvested and the freshly sub-cultured cells started at the next light intensity point. For the temperature experiments the cells were grown in a glass water tank in the cold room with a circulating heater to maintain the desired temperature. Incandescent bulbs were placed at the correct distance to give the required, constant light intensity. Again the cells left after the third sub-culture were harvested, the temperature lowered by 2°C and the process repeated. Due to its thermotolerant nature, *Rps. cryptolactis* was grown at high or low light in a circulating water bath at 38-40°C.

### 2.2.2 Chemoheterotrophic growth of Rhodospirillaceae.

A single well spaced photoheterotrophic colony was used to inoculate 25ml of media in a 250ml conical flask and incubated in a Gallenkamp orbital shaker at 30°C, 200 rev/min. After 1-4 days, depending on the species, the cells were of sufficient density to be harvested. Chemoheterotrophic cultures were never sub-cultured due to the ease of adventitious contamination.

### 2.2.3 Chemoheterotrophic growth of *E. coli.*

A single well spaced colony was used to inoculate LB-media in a conical flask and incubated in a Gallenkamp orbital shaker at 30°C, 200 rev/min. The volume of media never exceeded 10% of the flask volume.

## 2.3 Cell counting.

Calibration curves of cell number versus Optical Density (OD) at 650nm were determined and shown in Appendix B. From the starting point ($OD_{650}$=1), a dilution series was prepared, at each dilution the optical density was measured and the number of cells present estimated by counting on a haemocytometer slide. This necessitated immobilising/killing the cells by further dilutions in a solution of glycerol/iodine 1:1 (v/v).

Curves of *E. coli* cell numbers versus Optical Density at 600nm were determined by a different method (Sambrook *et al.*, 1989). A flask with 25ml LB-media was inoculated with a single colony and incubated with vigorous shaking at

37°C At intervals 1ml aliquots were removed, the $OD_{600}$ measured and serial dilutions prepared, these were then plated on LB-plates and incubated overnight at 37°C: Counting of the resultant colonies enabled the curves to be plotted.

## 2.4 Determination of antibiotic sensitivity.

All antibiotics used in this project were purchased from Sigma Chemical Company, Poole, Dorset and are given in Table 2.1. The stock solutions were filtered through a 2.2µm filter and stored at -20°C until required.

In order to ascertain the antibiotic concentration necessary for the forthcoming mating experiments minimal-Succinate plates (Appendix A) were prepared containing 0 (control), 1, 10, 20, 35, 50 or 100µg/ml of the desired antibiotic. These were then split into six segments. An aerobic culture of the cells was diluted to give $10^8$ cells/ml and 10-fold dilutions prepared. Onto the first segment 10µl of the $10^0$ was pipetted i.e. $10^6$ cells, onto the second 10µl of the $10^{-1}$ dilution was pipetted i.e., $10^5$ cells and so on for all six segments.

**Table 2.1 Antibiotic stock solutions.**

| Antibiotic | Solvent | Stock Concentration |
|---|---|---|
| Ampicillin anhydrous | $H_2O$ | 5 mg/ml |
| Streptomycin sulphate | $H_2O$ | 20 mg/ml |
| Kanamycin monosulphate | $H_2O$ | 25 mg/ml |
| Neomycin sulphate | $H_2O$ | 30 mg/ml |
| Tetracycline hydrochloride | $H_2O$:Ethanol (1:1) | 12.5 mg/ml |

The plates were incubated aerobically for 48 hours before being placed in an anaerobic jar for a further 4-5 days growth. Due to the debilitating effects of the antibiotics, especially at higher concentrations, it became difficult to count accurately the resultant colonies, therefore each segment was carefully scraped off with an inoculating loop, resuspended in 1ml of media and the number of cells present determined by measuring the $OD_{650}$. Comparison with the control plate enabled curves of Antibiotic Concentration (µg/ml) versus % Survival to be plotted.

## 2.5 Mobilisation of cloning vectors.

The method for conjugal gene transfer into *Rb. sphaeroides* was provided by Dr. C. N. Hunter, University of Sheffield and based on the mobilising *E. coli* strains and plasmids developed by Simon *et al.*, (1983). This was subsequently adapted for other members of the Rhodospirillaceae in this study. The experimental objective was to establish a reliable, reproducible method of introducing a gene of choice, initially one conferring antibiotic resistance, into the bacterial strain and not to determine exhaustively the conditions necessary to achieve optimum transfer efficiency. Therefore at the outset the following parameters were standardised; the recipient:donor ratio was 20:1 (di-parental matings) or 20:1:1 (tri-parental matings), unless otherwise stated in the Results, this in turn limited the incubation time to 6 hours in order to avoid excessive *E. coli* overgrowth. For this study all the members of the Rhodospirillaceae mentioned in 2.1 were used in the mating experiments, with the exception of *Rps. cryptolactis.*

### 2.5.1 Di-parental matings.

The Rhodospirillaceae species to be mated (see Fig 1.13) was grown chemoheterotrophically and immediately prior to the mating its $OD_{650}$ measured. Similarly, a few *E. coli* colonies were resuspended very gently in 50µl LB-media, microfuged for a few seconds, the supernatant discarded and resuspended very gently in 500µl fresh LB-media to remove any lingering antibiotic, after which the $OD_{600}$ was measured. Thus the number of recipient cells in 45µl and donor cells in

5µl could be determined from the graphs in Appendix B. The dilution of donor cells necessary to give the desired ratio of recipient:donor i.e., 20:1 is readily calculated. This mating mix i.e. total volume 50µl, was spotted onto a well dried LB-plate minus antibiotic and the liquid evaporated in a flow hood. Once dry (approximately 15min), the plate was incubated for 6 hours at 37°C. After which time the cells were scraped off and resuspended in a volume of minimal-Succinate medium, this was dependent on which plasmid was being transferred.

(a) Plasmids RP1, $pNH_2$ and pRK404 transferred with efficiencies of the order $10^{-1}$-$10^{-3}$ therefore cells were resuspended in 1ml, serial dilutions prepared and 20µl of each plated.

(b) The frequency with which kanamycin resistant ($Km^r$) colonies appeared after mating with the Tn5 harbouring plasmid pSUP2021 was of the order $10^{-6}$, therefore, cells were resuspended in 200µl, and 100µl plated.

The control plates were; photoheterotrophic or chemoheterotrophic cells on minimal-Succinate plates plus or minus antibiotic. In order to let the cells recover and express antibiotic resistance aerobic incubation was continued for approximately 48 hours at 30°C before placing the plates in an anaerobic jar. The transfer efficiency is given as the number of drug-resistant *trans*-conjugants obtained relative to the number of recipients recovered.

2.5.2 **Tri-parental matings.**

These were very similar to di-parental matings except the mating mix is composed of 40µl chemoheterotrophic Rhodospirillaceae, 5µl donor A and 5µl donor B (see Fig 1.14). After incubation the cells were resuspended in 1ml minimal-Succinate medium and spread on plates containing antibiotic, resistance to which is conferred by the plasmid harboured in donor B. Once removed from the anaerobic jar the plates were dried in a flow hood, then replica-plated onto plates containing the antibiotic resistance which is conferred by the plasmid corresponding to donor A, then to fresh donor B plates as a control and returned to an anaerobic jar for

colony regeneration. After approximately one week the colonies could be counted and the transfer frequency determined.

## 2.6 Standard molecular biological techniques.

In this section to avoid reproducing large established protocols, wherever possible, only alterations and modifications from the reference will be given.

### 2.6.1 Estimating DNA concentrations.

DNA concentrations were determined either by using the formula;

$$2 \times (A_{260} - A_{280}) \times \text{dilution factor}$$

with an OD=1 corresponding to approximately 50µg/ml double stranded DNA. Alternatively if only a small volume of DNA solution was available, then 1µl could be spotted on an Ethidium plate and the intensity of fluorescence when placed on a transilluminator compared against that emitted from known standards

### 2.6.2 Restriction digests.

All restriction enzymes were purchased from Gibco BRL and digests carried out according to manufacturers instructions.

### 2.6.3 Agarose gel electrophoresis.

Agarose gels with 1xTAE (Appendix A) buffer, containing 50µg/ml Ethidium bromide (EthBr) were poured and run according to Sambrook *et al.*, (1989), [pps 6.1-48]. Unless mentioned otherwise the percentage agarose was 0.8.

### 2.6.4 Preparation of radiolabelled lambda markers.

To enable molecular weight estimation of bands on autoradiographs the "5' DNA Terminus Labelling System" was purchased from Gibco BRL The Forward reaction was used to incorporate the radioactive phosphate group.

### 2.6.5 Plasmid purification.

(i) Large scale amounts of plasmid were prepared by the 'Alkali lysis' (Sambrook *et al.*, 1989, pps 1.38-9) method using a Beckman JA-14 rotor. The final nucleic acid pellet was dissolved in 8ml TE (pH8.0) [Appendix A] enabling a CsCl gradient to be run using a Sorvall UTB65 ultracentrifuge, T865.1 rotor (45,000rpm,

36h, 20°C). After the plasmid band was recovered, EthBr was removed by extraction with water-saturated butanol and dialysed against several changes of TE (pH8.0). Waste EthBr was decontaminated by the $KMnO_4$ method (Quillardet and Hoffnung, 1988).

(ii) Small scale preparations were performed by the 'boiling' method (Sambrook *et al.,* 1989, pps 1.29-30). The STET buffer recipe was provided by Dr. K. Kaiser, University of Glasgow; 8% Sucrose, 3% Triton X-100, 50mM EDTA, 50mM Tris.Cl (pH8.0).

### 2.6.6 **Transformation of *E. coli.***

This procedure was performed using the $CaCl_2$ method (Sambrook *et al.,* 1989, pps 1.82-84) which in turn is a variation of that of Cohen *et al* (1972). A separate set of glassware was kept for growing competent cells and never washed with detergent. DNA uptake occurred in 15ml Falcon polypropylene tubes which required a heat-shock at 42°C, determined empirically, of 90s duration.

### 2.6.7 **Preparation of lambda DNA**

*E. coli* NM621 plating bacteria and λ6 DNA were prepared as outlined in Sambrook *et al.,* (1989), [pps 2.118-20].

### 2.6.8 **Southern blotting.**

DNA was transferred from agarose gels to nylon filters using the procedure described by Southern (1975). The gel to be blotted was trimmed of all excess agarose, then pre-washed with excess 1.5M NaCl, 0.5M NaOH denaturing solution (3 x 20min), 0.25M HCl (2 x 15min) and 1.5M NaCl, 0.5M Tris.Cl (pH7.2), 10mM EDTA neutralising solution (1 x 60min). The DNA was transferred onto Hybond-N nylon filters, with 6 x SSC (Appendix A), overnight and fixed by 2min irradiation on a medium wavelength transilluminator (312nm).

### 2.6.9 **Preparation of radiolabelled probes.**

Labelled probes were prepared according to the 'Random Priming' method of Feinberg and Vogelstein (1983), unincorporated nucleotide was separated from the

probe by passage down a Sephadex G-50F 1ml column. Two of the probes that were prepared by this method require further explanation.

(1) The 3.3kb HindIII central fragment of Tn5 (see Fig 1.15a). This had been cloned into pBR322 and the resultant plasmid termed pKan2. To avoid continually referring to 'the 3.3kb HindIII Tn5 fragment from pKan2' in the context of radiolabelled probes, the name pKan2 alone will refer to this fragment.

(2) A 0.6kb PstI-BamHI fragment cloned into pUC18 to give plasmid pLHIISB18; the insert contains the *Rb. sphaeroides* pucA and pucB genes which encode the B800-850 light harvesting polypeptides. As above, in the context of radiolabelled probes the name pLHIISB18 alone will refer to this fragment. This plasmid was originally a kind gift from Prof. S. Kaplan.

2.6.10 **Hybridisation conditions**

This was performed using the Standard Hybridisation Procedure (Mason and Williams, 1985) and the composition of all hybridisation solutions is given in Appendix A. The double stranded probe was denatured by adding $^{1}/_{10}$ original volume 3M NaOH and left at room temperature (RT) for 5min, then neutralised with $^{1}/_{5}$ original volume of 1M Tris.Cl (pH7.5), followed by $^{1}/_{10}$ original volume 3M HCl.

The hybridisation conditions and wash regime afterwards depend on the sequence homology between the probe and the target sequence. When pKan2 was used to identify Tn5 genomic insertions induced by plasmid pSUP2021, this relationship is 100%, therefore, a high stringency wash regime was used i.e.

3 x for 20 min in 3 x SET, 0.1% SDS, 0.1% NaPPi at 68°C.

2 x for 20 min in 1 x SET, 0.1% SDS, 0.1% NaPPi at 68°C.

1 x for 20 min in 0.1 x SET, 0.1% SDS, 0.1% NaPPi at 68°C.

1 x for 20 min in 4 x SET at RT.

When pLHIISB18 was used to probe *Rps. acidophila* DNA, it was necessary to use a less stringent regime This was determined empirically to be:

3 x for 20 min in 3 x SET, 0.1% SDS, 0.1% NaPPi at 60°C.

2 x for 20 min in 1.5 x SET, 0.1% SDS, 0.1% NaPPi at 60°C.

1 x for 20 min in 4 x SET at RT.

If filters were to be re-probed, it was important not to let them dry out after the final wash. To remove the first probe the filters were washed with 30mM NaOH (10min, RT) with gentle agitation, then immersed in 10mM Tris.Cl (pH 7.5), 1mM EDTA (10min, RT) again with gentle agitation. This was repeated twice more then exposed to x-ray film to ensure all the probe had been removed.

2.6.11 **Generation of nested deletions using exonucleaseIII**

These were created using the protocol given in Sambrook *et al.,* (1989) [pps 13.39-41], with the slight simplification that the DNA from each 30s timepoint was blunt-end ligated using one Weiss unit of T4 DNA ligase (Gibco BRL and their buffer).

2.6.12 **Polymerase chain reaction (PCR).**

Two 20mer PCR primers were designed that had a G+C content of 60% which meant the annealing step was performed at 55°C. $10^9$ DNA molecules were amplified through 40 cycles using an 'Autogene' PCR machine manufactured by Grant.

## 2.7 Preparation of high molecular weight genomic DNA.

This procedure was modified slightly from that provided by L. Gibson, University of Sheffield and another by Dr. R. C. MacKenzie, University of Glasgow, with reference to that of Birnboim and Doly (1979) and Klug and Drews (1984)

Late-log phase cells (100ml) were harvested and washed twice with 10ml ice-cold STE (Appendix A) and resuspended in 10ml STE. 2ml of freshly made up lysozyme (10mg/ml) was added and incubated for 10min at 37°C. However, it was discovered that *Rps. acidophila* required incubating for up to 24h to enable sufficient

cell wall digestion to occur. An equal volume of STE + 2% SDS + RNase (20µg/ml) was added and incubated at 42°C for 1h. Once again *Rps acidophila* required up to 24h. Proteinase K was added to a final concentration of 50µg/ml and incubated at 55°C until the solution became translucent. An equal volume phenol/chloroform was added (i.e., approx. 24ml) and rolled gently at room temperature for 1h. To separate the phases the mixture was centrifuged (5000g, 1h, 4°C), then the upper aqueous, DNA containing phase was removed into a clean Falcon tube using a wide-bore pasteur pipette avoiding denatured protein at the interface. This extraction/centrifugation procedure was repeated until the protein interface was no longer visible, enabling the DNA solution to be finally extracted with chloroform. The upper aqueous phase was again removed to a fresh Falcon tube, whereupon $^1/_{10}$vol. 3M Na. acetate (pH5.2) and 2½vol. ice-cold ethanol were added, mixed gently and placed at -20°C. The precipitate was removed using a 'hooked' Pasteur pipette, washed with 70% ethanol, air-dried (5min) and carefully teased into 1ml TE (pH8.0) added. The DNA was allowed to dissolve on the bench for as long as was necessary (good quality, high molecular weight DNA should take 2-3 days ).

## 2.8 **Colony lysis.**

For Rhodospirillaceae, this protocol was provided by Dr C. N. Hunter, University of Sheffield and adapted as necessary.

Putative Tn5 containing colonies were toothpicked into a 5x5 array on kanamycin minimal plates, firstly to a master plate then onto plate overlaid with a Hybond-N filter. The colonies were regenerated by growth in an anaerobic jar. After 4-5 days ,the filter was peeled off the plate with forceps, control DNAs pipetted onto the filter and laid colony side up on a stack of Whatmann 3MM paper saturated with, in turn: denaturation solution (1.5M NaCl, 0.5M NaOH) for 3 min and allowed to dry, neutralisation solution (1.5 M NaCl, 0.5 M Tris.Cl [pH 7.4]) for 5 min and then 3 x SSC for 30 sec. Finally the filters were transferred to dry 3MM paper and allowed to dry at RT. The DNA was fixed to the filter by baking in an vacuum oven at 80°C for 3

hr. Attempts to fix the DNA using UV-irradiation were unsuccessful To remove the adhering cell debris the filters were pre-washed in 0.1% SDS, 3 x SSC at 65°C overnight. For *Rps. acidophila* the time for denaturation was extended to 30 min. All master plates were stored at 4°C

For *E. coli* the procedure was identical except prior to denaturation the filter was laid on 3MM paper saturated with 10% SDS and the final pre-wash omitted.

## 2.9 DNA sequencing

The set of nested deletions were sequenced using the dideoxy chain termination method of Sanger *et al.,* (1977). The sequencing reactions were performed using a Sequenase® Version 2.0 kit supplied by United States Biochemical and in accordance with the manual. For the annealing step 1pmol primer per 4µg double stranded plasmid template was used and the gel run for 2-4h depending on the region if the template to be resolved. All solutions for sequencing are given in Appendix A.

The gel plates were cleaned with detergent and allowed to air dry, whereupon the inside face was cleaned with methanol, then isopropanol. The smaller plate was then treated with Dimethyldichlorosilane solution (BDH) in a fume hood and allowed to air dry for 10min after which it was washed twice with methanol. The two plates were assembled, using plastic spacers for a 0.4mm flat gel or 3mm paper spacers for a wedge gel, then a 6% acrylamide solution in 7M urea poured between them. After polymerisation the gel was pre-run (65W, 1500V, 35mA) for 1h. Immediately before loading samples the wells were rinsed thoroughly with running buffer i.e., 1 x TBE.

After the gel had been run the siliconised small plate was removed and the gel immersed in 3l of 10% methanol, 10% acetic acid for 15min (flat gel) or 30min (wedge gel), then transferred to 3MM paper and dried at 80°C for up to 3h whereupon the gel was exposed as necessary.

## 2.10 Preparation of chromatophores.

Chromatophores were prepared essentially as described by Cogdell *et al.*, (1983). The pertinent cells were harvested with a low speed spin (5000g, 100min, 4°C) and resuspended in 30ml ice-cold 20mM MES/KCl (pH 6.8), homogenised and a small amount of $MgCl_2$ and DNase added, prior to one passage through the French Press at 950 p.s.i. Unbroken cells and large cell debris were removed with a low speed spin (4000g, 10min, 4°C). To pellet the chromatophores they were ultra-centrifuged in a Sorvall OTD-55 (50,000 rpm, 1h, 4°C) then resuspended in a minimal volume of 20mM Tris.Cl (pH 8.0). The supernatant was homogenised and standardised to an $OD_{590} = 25cm^{-1}$.

## 2.11 Bacteriochlorophyll assay.

Different volumes of chromatophores (5µl, 15µl, 30µl) were made up to 1ml with acetone:methanol (7:2) in Eppendorf tubes protected from the light, vortexed and microfuged for 5min, with the supernatant removed to fresh tubes. Care was taken to keep the extract dark at all times. An absorption spectrum between 800nm and 700nm was recorded and the absorbance maxima at approx. 772nm noted, this was used to calculate the [BChl] using the *in vitro* extinction coefficient (ε) of 76 $mM^{-1}cm^{-1}$ (Clayton, 1980).

## 2.12 Solubilisation of chromatophores.

A 10%($^{w}/_{v}$) solution of each of the detergents n-dodecyl-ß-D-Maltoside (DDM) and n-octyl Glucoside (BOG) were prepared. From the extinction coefficient, the amount (g) of Bchl in any given volume of chromatophores could be calculated and therefore the correct volume of detergent added to give the desired detergent/Bchl ratio e.g., 35:1 is equivalent to 17.5DDM:17.5BOG:1Bchl. The mixture was vortexed hard (1min) and microfuged (5min) to pellet any unsolubilised material, and the supernatant of solubilised chromatophores was removed to a fresh tube.

## 2.13 Polyacrylamide gel electrophoresis (PAGE).

Gels were run using the Atto Vertical PAGE System apparatus purchased from Genetic Research Instrumentation Ltd., Essex. The compositions of all PAGE solutions are given in Appendix A.

### 2.13.1 Deriphat-PAGE.

The surfactant Deriphat 160 (N-lauryl-ß-iminoproprionate) was purchased from Henkel, Minneapolis. This fractionation system resolves antenna complexes in their intact state, giving increased resolution and stability compared to previous systems (Ferguson *et al.*, 1991). After gel polymerisation, the apparatus was placed at 4°C for pre-cooling whilst the samples were prepared. To 50µl solubilised chromatophores one drop glycerol is added, vortexed, and 20µl loaded onto the gel and run at 20mA for approx. 40min at 4°C.

### 2.13.2 SDS-PAGE

This gel was run according to the method of Laemmli (1970). A 1mm 10-20% gradient gel was poured with a 3% stacking gel. The samples were prepared by standardising the $Q_y$ band to an $OD_{890}=1cm^{-1}$, adding an equal volume Boiling Solution, boiling for 3min and placing on ice prior to loading. The gel was run at 30mA through the stacker and thereafter at 8mA overnight. The standards were BSA (66kD), alcohol dehydrogenase (40kD), myoglobin (17kD), cytochrome c (12kD) and the gel developed by the silver stain method of Morrisey (1981), except that 12ml of 2.3M citric acid was added per 100ml developer to stop the staining reaction.

## 2.14 Electron microscopy.

The electron micrographs presented in this project are the work of Mr E. Robertson, EM suite, University of Glasgow. The cells were fixed in a 0.2M phosphate buffer and photographed with a Zeiss EM902 Transmission Electron Microscope.

## 2.15 Fluorescence experiments.

The growth of *Rps. acidophila* and *Rps. cryptolactis* cells and preparation of chromatophores was at the Department of Botany, University of Glasgow. All spectrofluorimetric experiments were performed at the Department of Biophysics, Huygens Laboratory, University of Leiden, Leiden, Netherlands.

The precise configuration of the spectrofluorimeter is described in Deinum (1991). The wavelength of the exciting laser flash was 532nm with a flash duration of approx. 25ps. By means of a variable neutral density filter the energy incident on the sample could be adjusted between $10^{-5}$ and 5mJ/cm$^2$. Fluorescence emission spectra were recorded with the same apparatus, with excitation by either the laser flash or by low intensity xenon flash. In the latter case the light was filtered by means of a combination of filters yielding a band with a width of 40nm, centred at 515nm. The monochromator for detecting the fluorescence was set at a bandwidth 1.5nm for the measurement of fluorescence spectra, and at 4nm when annihilation at a fixed wavelength was measured. Both the excitation energy and the relative fluorescence were measured with photodiodes. The absorbance of the samples was <0.05 (at 532nm) in order to achieve homogeneous light distribution within the sample and to limit the degree of self absorption of fluorescence. Changes in the redox state of the RC were brought about by continuous background light filtered by a Schott BG-38 filter. All measurements were performed at room temperature.

## 2.16 Carotenoid extraction from chromatophores.

This procedure was provided by Dr G. Britton, University of Liverpool. To approx. 1ml chromatophores in an unused test tube an equal volume acetone and equal volume petroleum ether (40-60°C) [Pet. ether] were added and vortexed for 30s, approx. 5ml distilled water was added and re-vortexed, whereupon the carotenoids partition into the upper hydrophobic phase. These are then removed with a Pasteur pipette to a fresh tube. More acetone / Pet. ether can be added and the process repeated until all or most of the carotenoids have been removed,

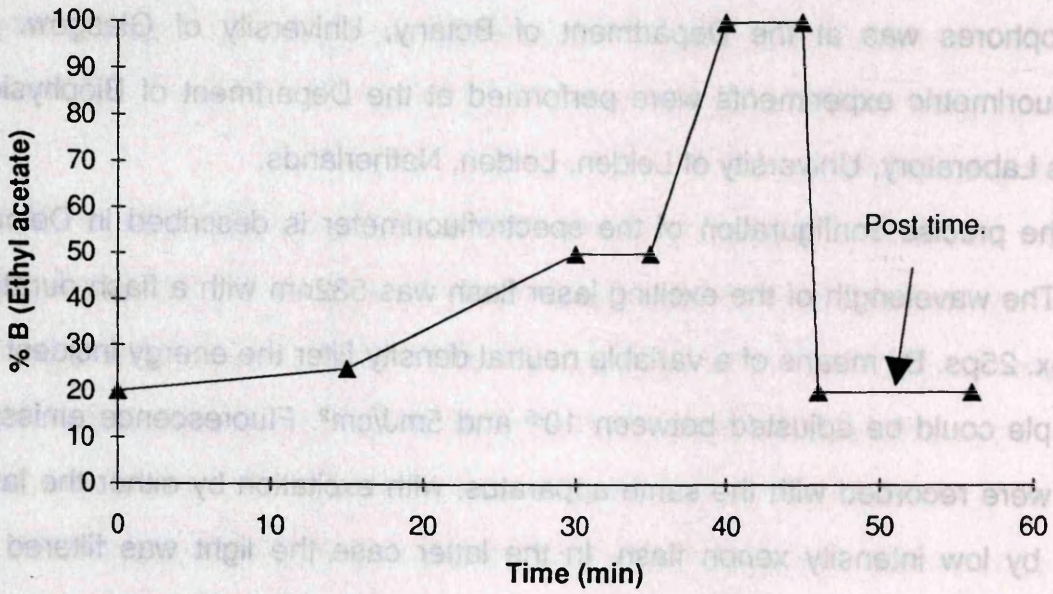



**Figure 2.1**: Gradient used to elute carotenoids off a $C_{18}$-octadecylsilyl (ODS) 'Spherisorb'® column. Other chromatographic parameters were;

Flow rate 1ml/min; oven temp 40°C; injection volume 50µl.

Solvent system: A, 10% water in acetonitrile; B, ethyl acetate

**Table 2.2 Diode array detector (DAD) parameter settings**

| DAD Parameter | Setting | Explanation |
|---|---|---|
| Store spectrum | Peak controlled | Spectra stored at upslope, apex, downslope of peak. |
| Threshold | 2.0 mAU. | Height of smallest peak expected |
| Peakwidth | 0.1 min | Half-height width of narrowest peak expected. |
| Sampling interval | 640ms | Time between two data points. |
| Spectrum range | 300 to 600nm | Spectra saved between these two wavelengths |

whereupon they were evaporated to dryness and kept under $O_2$-free $N_2$ until required. During the extraction procedure it is important to keep samples shielded from light to prevent electronic absorption and consequent formation of destructive singlet oxygen.

## 2.17 **High performance liquid chromatography (HPLC).**

Carotenoid samples dissolved in dichloromethane were filtered through a 0.45µm cellulose membrane and run using an Hewlett-Packard HP1090 Solvent Delivery System and 1040A Diode Array Detector. Initially it was intended to use the 'General Reversed Phase Gradient' to elute the carotenoids off the column. This gradient was provided by Dr G. Britton, University of Liverpool and is used successfully in his laboratory. With our apparatus, however, it was found that after completion of the run the most apolar carotenoids were still retained on the column, therefore, it was necessary to modify the gradient. Unfortunately due to time constraints this was only possible to a limited extent; the gradient used and the chromatographic parameters are given in Figure 2.1

The Diode Array Detector (DAD) [see Bramley, 1992] was set to record the resultant chromatographs (a plot of absorbance against time) and spectra (a plot of absorbance against wavelength) at three wavelengths; 473nm, the absorbance maxima of rhodopin and its glycoside, 510nm that of rhodopinal and its glycoside and 360nm the absorption maxima of the Bchl Soret band. Each was set with a bandwidth of 4nm. The other DAD parameters are outlined in Table 2.2. All solvents were HPLC grade and purchased from Rathburn Chemicals, Walkerburn, Scotland.

# Chapter Three

## Characterisation of the changes in the *Rps. acidophila* peripheral antenna when cultured under non-optimal conditions.

## 3.1 Introduction.

Two environmental signals are known to affect the peripheral antenna complement in *Rps. acidophila;* light intensity (I) and temperature (T). These stimuli modulate antenna complex (AC) formation and carotenoid biosynthesis in different ways for the three *Rps. acidophila* isolates used in this study. Full understanding of this regulatory process will only be gained through the application of a multidisciplinary approach, with emphasis on modern molecular biological techniques. A necessary first step in this direction is to fully characterise the changes in the *Rps. acidophila* photosynthetic unit when the growth conditions are altered, this data can then act as a benchmark from which individual components responsible for particular effects can be dissected away from the overall process.

Although post-transcriptional and possible post-translational processing is known to play a role in regulating the level of B800-850 complexes (Zucchini and Beatty, 1988), for the purposes of this chapter it will be assumed that such processing does not occur and any insertion of new complex is the direct result of *de novo* expression.

## 3.2 Effect of light intensity and temperature on the photosynthetic unit of *Rps. acidophila* strain 7750.

This strain is known to produce the B800-820 complex at low temperatures i.e., <24°C in preference to B800-850 (Cogdell, R. J., pers. comm.). The exact growth conditions, however, which promote this change have not been characterised in any detail. Three different growth regimes were investigated;

(1) Lowering the incident light intensity on the culture but keeping the temperature constant.

(2) Lowering the growth temperature of the culture whilst maintaining a relatively high incident light intensity.

(3) Lowering the growth temperature of the culture whilst maintaining a relatively low incident light intensity.

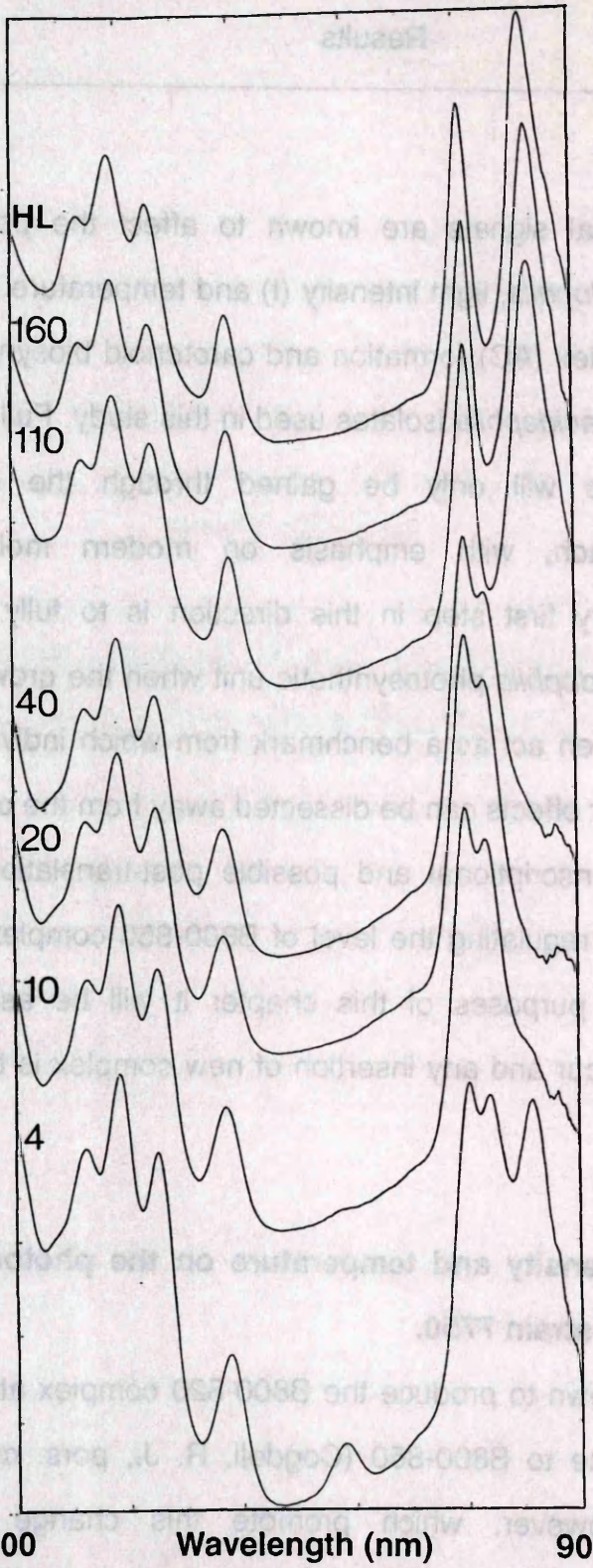



**Figure 3.1:** Spectra obtained by growing *Rps. acidophila* 7750 at progressively decreasing light intensities. The spectra series presented in this chapter follow the same pattern: The topmost spectrum is obtained when the species is cultured under 'normal' growth room conditions i.e., high light (approx. 160µmols/s/m² at 30°C) and serves as a benchmark from which any changes can be compared. The second topmost spectra is the first in the series and the conditions have been adjusted as far as possible to simulate the growth room i.e., the top two spectra should be identical. The spectra follow in order from top to bottom; the left-hand numbers indicate the light intensity (µmols/s/m²) at which that spectra was obtained.

For the first of these protocols, cells were harvested at different light intensities, French Pressed and the chromatophores solubilised as described previously (2.10). An absorption spectrum was recorded for each light intensity value and the results presented in series in Figure 3.1. From this figure it is apparent that strain 7750 produces B800-850 as its peripheral component under 'normal' growth room conditions, however, as the light intensity is lowered this is replaced by B800-820. This can easily be seen with the spectra corresponding to 110 and 40µmols/s/m$^2$, the former has NIR maxima at approx. 800 and 850nm, whereas the latter maxima are 800 and 820. Spectrophotometrically, there was no evidence of any difference in the carotenoid composition of the B800-820 or B800-850 spectra, although, visible inspection showed B800-850 containing cells were pale orange, whereas their B800-820 containing counterparts were a deep orange brown. A further, somewhat unexpected, observation was the appearance of a shoulder at 850nm in the 10µmols/s/m$^2$ spectrum, moreover in the 4µmols/s/m$^2$ spectrum this had developed into a full peak. At these extremely low light intensities this is possibly due to forced derepression of B800-850 genes, resulting in some form of desperation response enabling the cell to increase the cross-sectional area of the photosynthetic unit (PSU) thereby capturing more of the scarce photons. A similar PSU composition is noted when 7750 is grown <18$^{o}$C (A. Hawthornthwaite pers. comm.). This phenomena merits detailed investigation, however, it was not studied further as part of this thesis.

Until recently electrophoretic analysis of intact bacterial photosynthetic pigment-protein complexes has lagged behind those of higher plants, instead, separation of these extracts has utilised column chromatography (e.g., Cogdell, 1986). Column based methods were unsuitable in this case due to the relatively small amounts of cells harvested at each light intensity (approx. 0.2g cells wet weight). Fortunately, it has been found that the use of the surfactant Deriphat-160 effectively stabilises higher plant pigment-protein complexes during gel electrophoresis, preventing dissociation of the pigments from their apoproteins. This

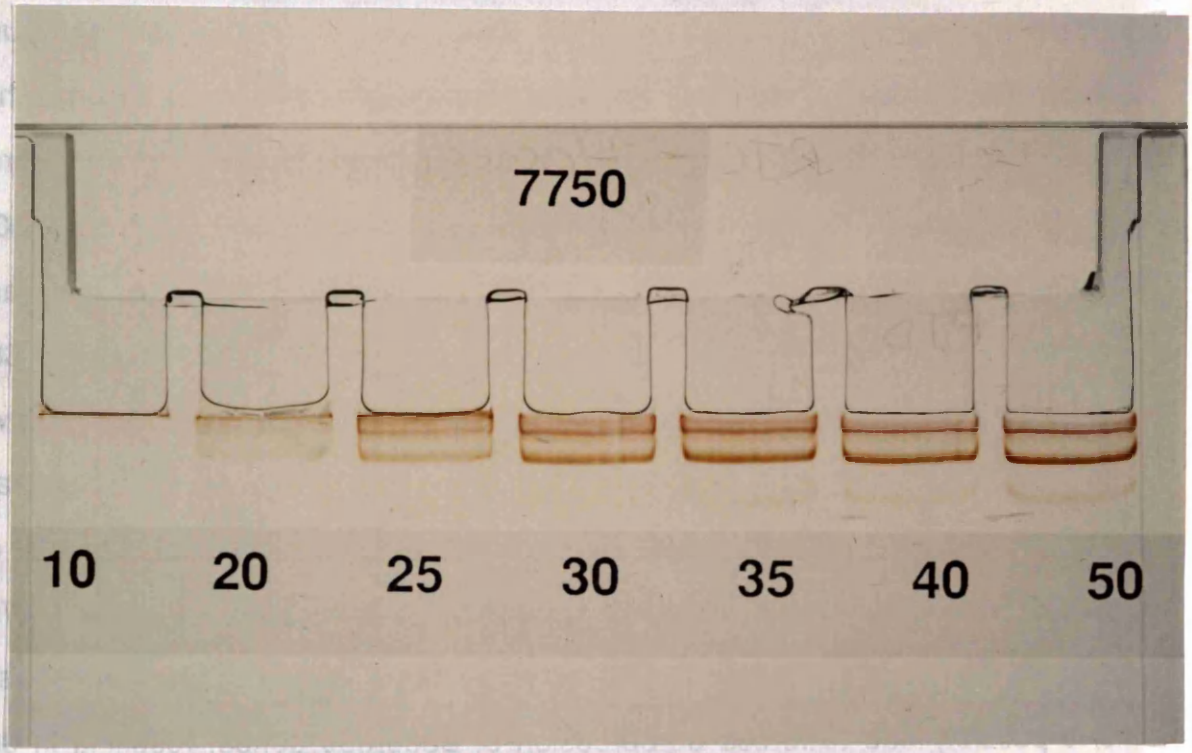



**Figure 3.2:** Deriphat-PAGE gel to determine the optimal detergent/Bchl ratio for solubilising *Rps. acidophila* 7750 chromatophores that yields the greatest resolution with this technique. The numbers indicate the parts detergent used in the respective well e.g., 35 means the chromatophores were solubilised with a detergent/Bchl ratio of 35:1.

technique has now been applied to bacterial complexes (Ferguson *et al.*, 1991), involving solubilisation of the chromatophores with a 1:1 ratio of two glycosidic detergents dodecylmaltoside (DDM) and ß-octylglucoside (BOG) and then separating them electrophoretically in the presence of Deriphat-160, providing a rapid, convenient, and importantly in this experimental context, a microscale procedure for analysing the photosynthetic apparatus with minimal disruption of individual pigment-proteins. Therefore, this technique was adapted for use as an assay to study variation in the composition of the photosynthetic unit of cultures grown under the different conditions of light intensity and temperature in this work.

Before this analysis is possible, it was necessary to determine the amount of detergent which in combination with Deriphat-160 results in optimum stabilisation of the pigment-protein complex during electrophoresis. This is achieved by assaying chromatophores for Bchl a content and adding increasing amounts of BOG and DDM to yield final solubilised chromatophores which have detergent:Bchl ratios ranging from 10:1 to 50:1. The Deriphat-PAGE gel in Figure 3.2 illustrates this experiment for *Rps. acidophila* 7750 chromatophores. It is evident that both the 10:1 and 20:1 ratios do not solubilise the complexes, presumably because there is simply not enough detergent present. The 25:1 has solubilised the complex but there appears also large molecular weight aggregates which have only partially entered the gel. The other four ratios have all solubilised the chromatophores and have been stabilised by the surfactant during the electrophoresis. The ratio 35:1 was deemed to be optimal and in addition this experiment was repeated for *Rps. acidophila* 7050 and 10050 yielding the same result; henceforth, all chromatophores prepared from different growth conditions were solubilised with this ratio.

A Deriphat-PAGE gel was run with the 7750 solubilised chromatophores that had provided Figure 3.1, i.e., decreasing light intensity, resulting in Figure 3.3. Two different types of complexes are apparent; the top band corresponds to the B890/RC conjugate and the bottom band to the more numerous B800-850 and/or B800-820 complexes. This Figure reveals that when the light intensity was lowered

from 160 to 110µmols/s/m², there was no difference in carotenoid composition of either complex. However, from 40 to 4µmols/s/m², the carotenoids present in the B800-820 complex are appreciably different, confirming the colour observations on whole cells intimated above. Because the pigment-protein complexes have been stabilised by Deriphat-160 during the electrophoresis, the bands can be excised from the gel and their spectra recorded. A caveat at this point is neccessary: although the complexes themselves are relatively stable the electrophoresis and excision should be done rapidly, and when possible with the exclusion of light to minimise production of destructive oxygen radicals. After spectra from all bands in Figure 3.3 were recorded they confirmed that the changes in Figure 3.1 were due solely to the peripheral complexes, i.e., removal of B800-850 and insertion of B800-820, the 'switch' occurring as the light intensity was lowered from 110 to 40µmols/s/m². Spectra from these two light intensities are presented in Figure 3.4. There was no difference in the spectra recorded for the B890/RC conjugate throughout the range of light intensities evaluated, confirming that this complex is the 'fixed' part of the antenna. The carotenoid composition present in the B890/RC and B800-850/B800-820 complexes at 110 to 40µmols/s/m² can be compared: what is immediately apparent is that the compositions of both types of complex are different irrespective of the light intensity. However throughout the range of this series, there was no detectable change in the carotenoid compositions of either complex.

The second growth regime, that of growing cultures at progressively lower temperatures whilst maintaining a relatively high light intensity (approx. equivalent to that in the growth room), was now investigated. The spectra recorded at each temperature are given in series in Figure 3.5. *Rps. acidophila* has been reported to be temperature sensitive with regard to its peripheral antenna i.e., B800-820 is formed only when cell growth occurs at less than 24°C. Initial inspection of the results obtained in Figure 3.5 appear to be at odds with these findings, even at 20°C the B800-850 complex is still being inserted into membrane. Closer scrutiny reveals

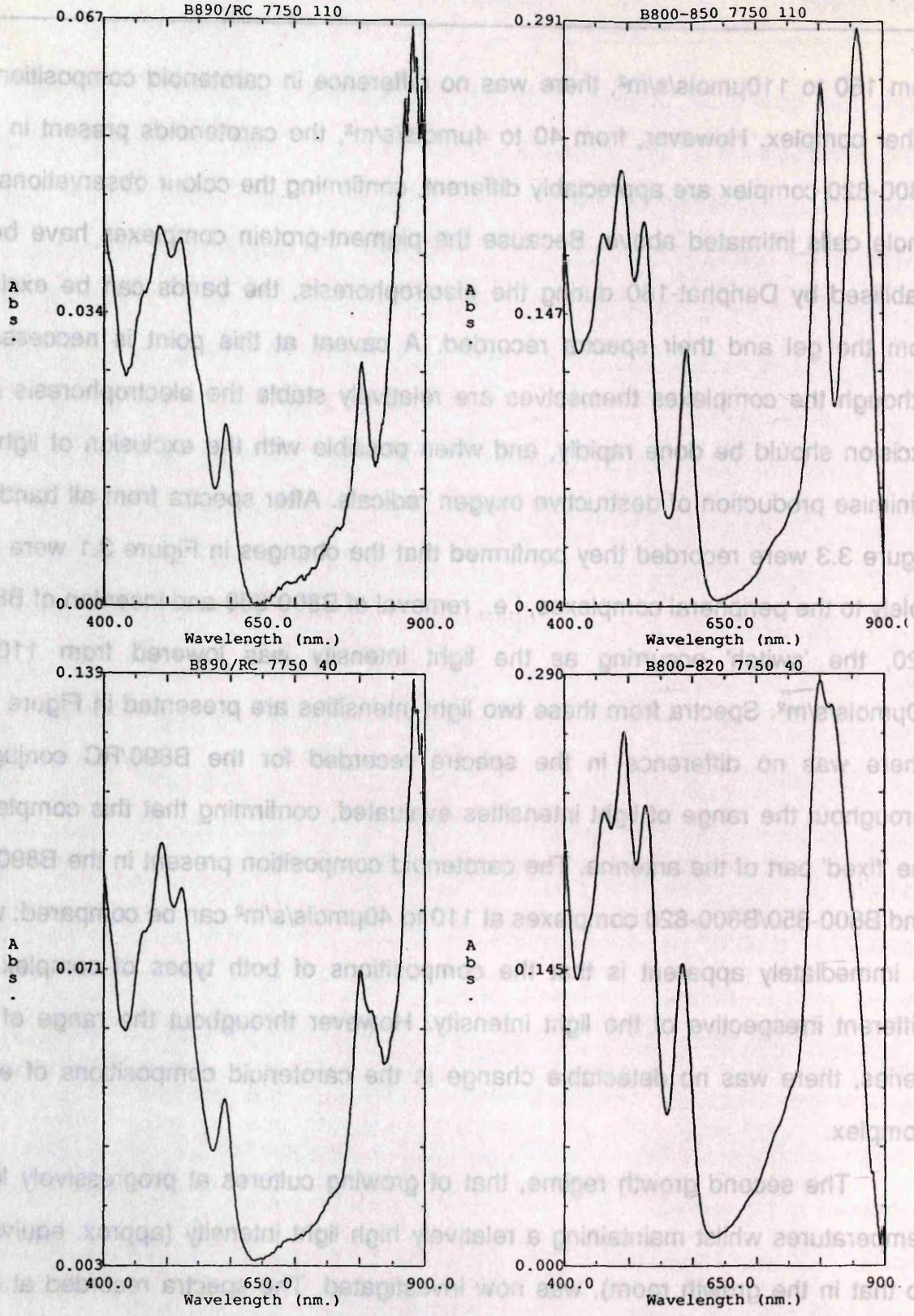



**Figure 3.4:** Spectra from excised bands, wells 110 (top row) and 40 (bottom row), obtained from the previous figure. The two left-hand spectra are the B890/RC conjugate and show no change as the light intensity is reduced, however, the right-hand spectra illustrate dramatically the change in the peripheral antenna as B800-850 is replaced by B800-820.

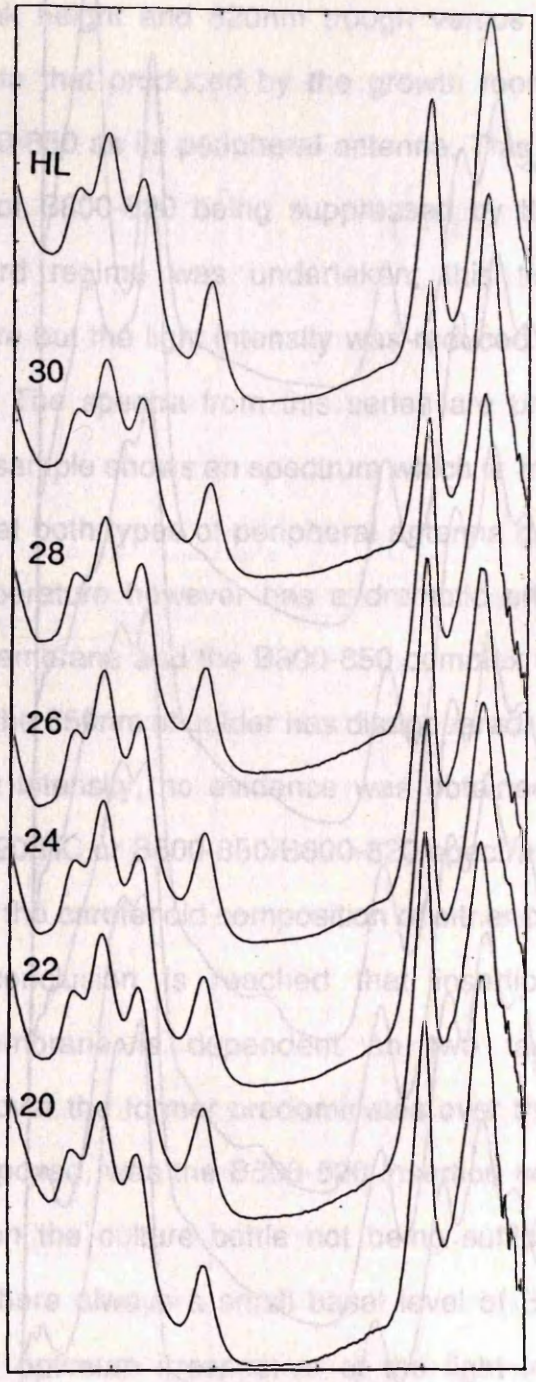



**Figure 3.5:** Spectra series obtained when *Rps. acidophila* 7750 is cultured at progressively decreasing temperatures (°C) in conjunction with a high light intensity (160µmols/s/m²). A basal level of B800-820 complex is evident.

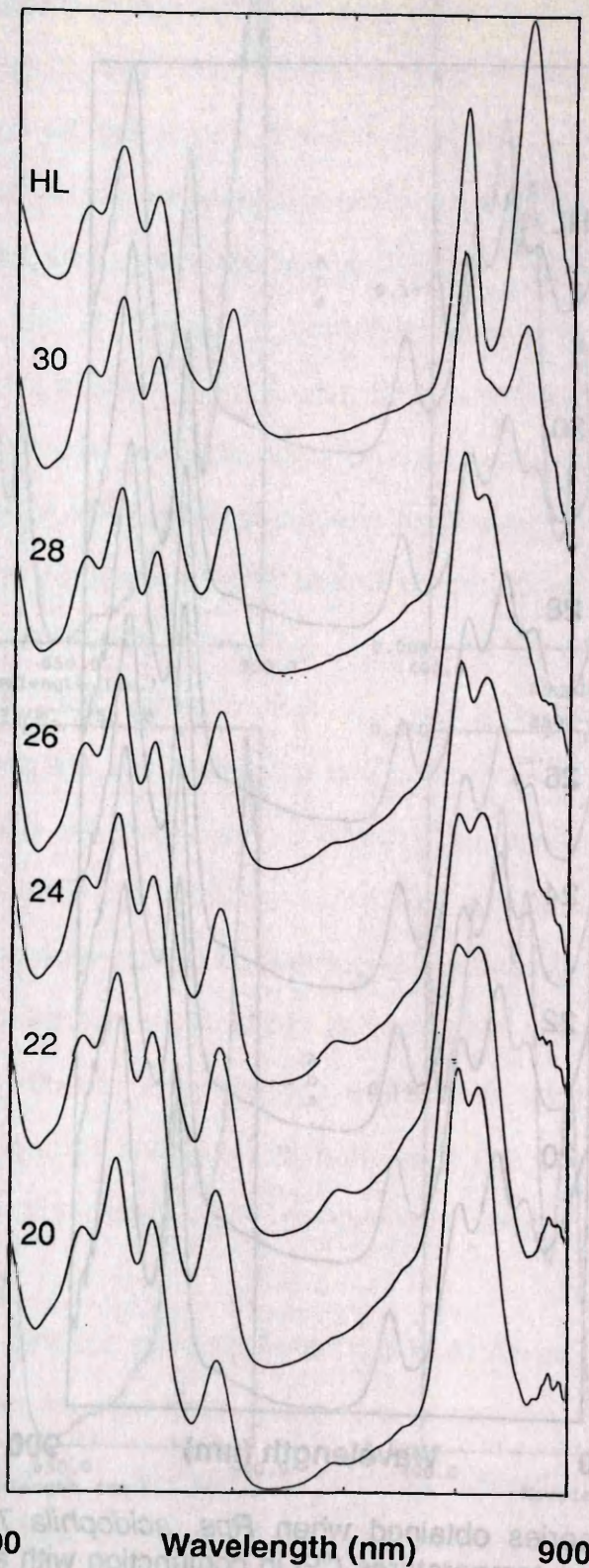



**Figure 3.6:** Spectra obtained when *Rps. acidophila* 7750 is cultured at progressively decreasing temperatures (°C) in conjunction with a low light intensity (approx. 50µmols/s/m²). In this case the second top spectrum is not expected to match the growth room spectrum as low light induced B800-820 complex is present.

that lower than 28°C there has been a small amount of B800-820 produced, judged by the 850nm peak height and 820nm trough versus the height of the peak at 800nm compared to that produced by the growth room, high light culture which produces only B800-850 as its peripheral antenna. This then begged the question: is the expression of B800-820 being suppressed by the high light intensity? To answer this a third regime was undertaken; this time the temperature was decreased as before but the light intensity was reduced to approximately a third of the previous level. The spectra from this series are presented in Figure 3.6. As expected the 30°C sample shows an spectrum which is 'intermediate' in that the light intensity is such that both types of peripheral antenna genes are being expressed. Reducing the temperature however has a dramatic effect: B800-820 complex is inserted into the membrane and the B800-850 complex is progressively diluted out until at about 22°C the 850nm shoulder has disappeared.

As with light intensity, no evidence was obtained from examination of the Deriphat-PAGE B890/RC or B800-850/B800-820 spectra that temperature is able to induce a change in the carotenoid composition of either complex.

Thus the conclusion is reached that insertion of B800-820 into the photosynthetic membrane is dependent on two factors; light intensity and temperature, moreover the former predominates over the latter. If this is correct a further question is posed, was the B800-820 insertion noticed in Figure 3.5 due to the incident light on the culture bottle not being sufficient to totally prevent this phenomena or is there always a small basal level of B800-820 when the growth temperature is not optimum irrespective of the light intensity. Reference to the Methods section (3.1) reveals these experiments were performed by growing the cells in a water filled glass tank in a cold room illuminated with banks of bulbs to provide the requisite light intensity and a heater to provide the desired temperature. To test this latest hypothesis the heater was removed and the banks of bulbs moved as close as possible to the tank, this had the effect of maximising the light

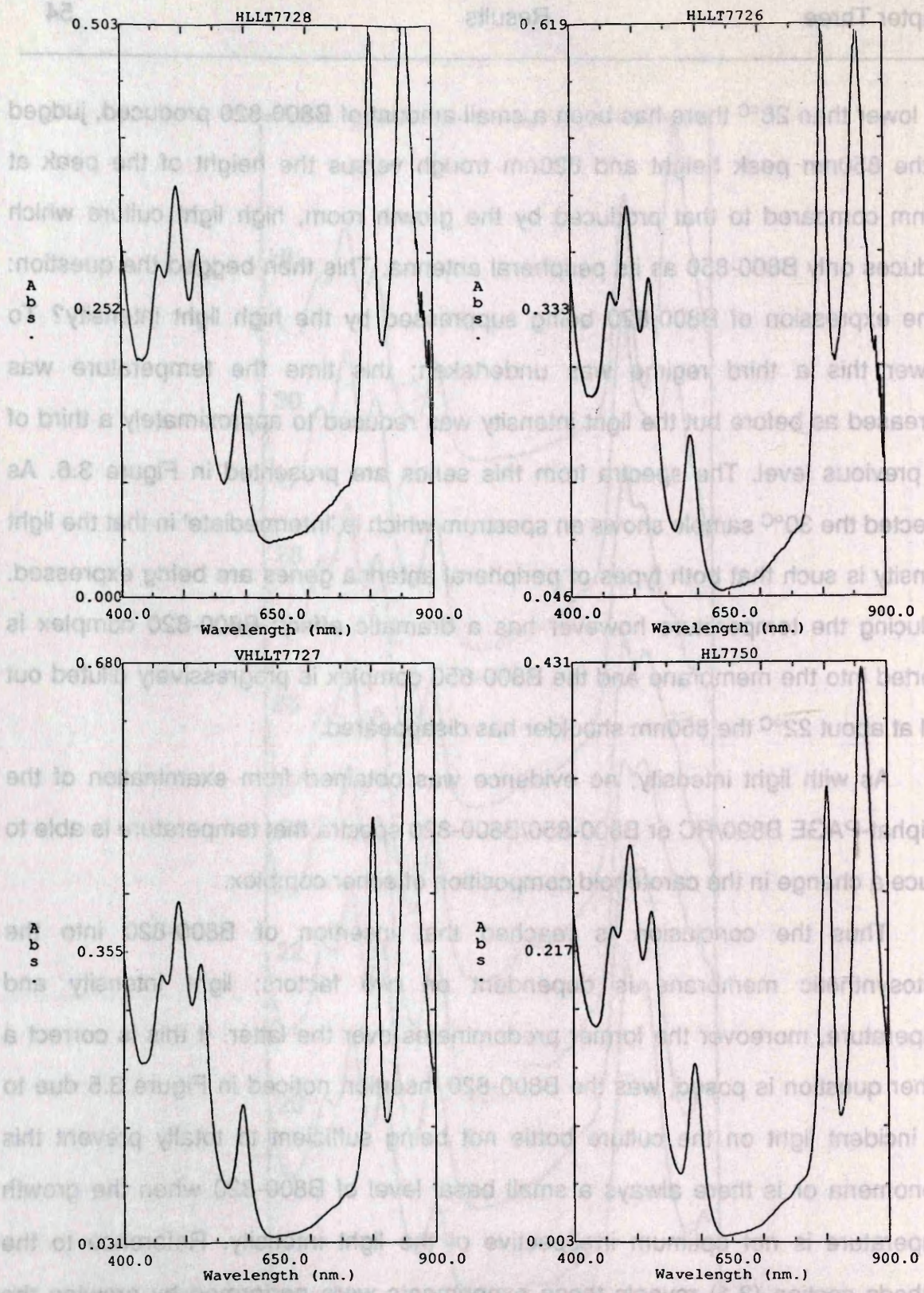



**Figure 3.7:** Experiment to prove *Rps. acidophila* 7750 B800-820 expression is primarily regulated by light intensity. The upper two spectra are reproduced from Fig. 3.5 (28°C [left] and 26°C [right]) and show a small amount of B800-820 present. The bottom left spectrum shows the same culture grown at the maximum possible light intensity under these conditions (and at 27°C). The high light intensity has suppressed the expression of the B800-820 genes and all the peripheral antenna is now B800-850. For comparison, the same culture (bottom right) was also grown under normal growth conditions which is known to produce only B800-850.

and heating the water to 27°C. The spectrum recorded at this temperature is presented in Figure 3.7c.

Figures 3.7a and 3.7b are reproduced from Fig. 3.5 and show clearly the small amount of B800-820 present, whereas 3.7c is equivalent to the growth room spectrum of 3.7d i.e., only B800-850 is being produced, and the expression of B800-820 can be absolutely suppressed if the light intensity is sufficiently large. This suggests that the light intensity, when the cells were grown for Fig 3.5, was in fact not quite sufficient to totally suppress B800-820 expression, even though theoretically it was adjusted to be the same as in the growth room, this can be understood when it is remembered that precise measurement of incident light intensity on a bottle is a complex mathematical problem and not simply a case of pointing a light meter in the direction of the bulb.

Figure 3.8 illustrates that antenna complex composition within the PSU, and the control pathways which regulate its expression, are not to be envisaged as in any way fixed, rather they are in a state of dynamic flux, responding to the environmental stimuli in such a way as to continually optimise the cells light harvesting capability.

When the 22°C culture (Figure 3.8a) from Fig. 3.5 was grown under three other possible growth conditions the PSU altered rapidly, with the cells adapting to their new environments; when the light intensity was reduced but the growth temperature was still 22°C (Fig. 3.8b), B800-820 was expressed, similarly, when the cells were grown at low light but at 30°C (Fig. 3.8c) B800-820 again was expressed. Finally, the same culture was grown in the growth room under high light, B800-820 was not required and B800-850 is the only peripheral component present (Fig. 3.8d).

In summary, the peripheral antenna complex composition of *Rps. acidophila* strain 7750 is regulated by a light intensity and temperature 'switch', the latter is subordinated, and as such can be repressed by the former. Regulation of carotenoid biosynthesis does not appear to be affected by these stimuli.

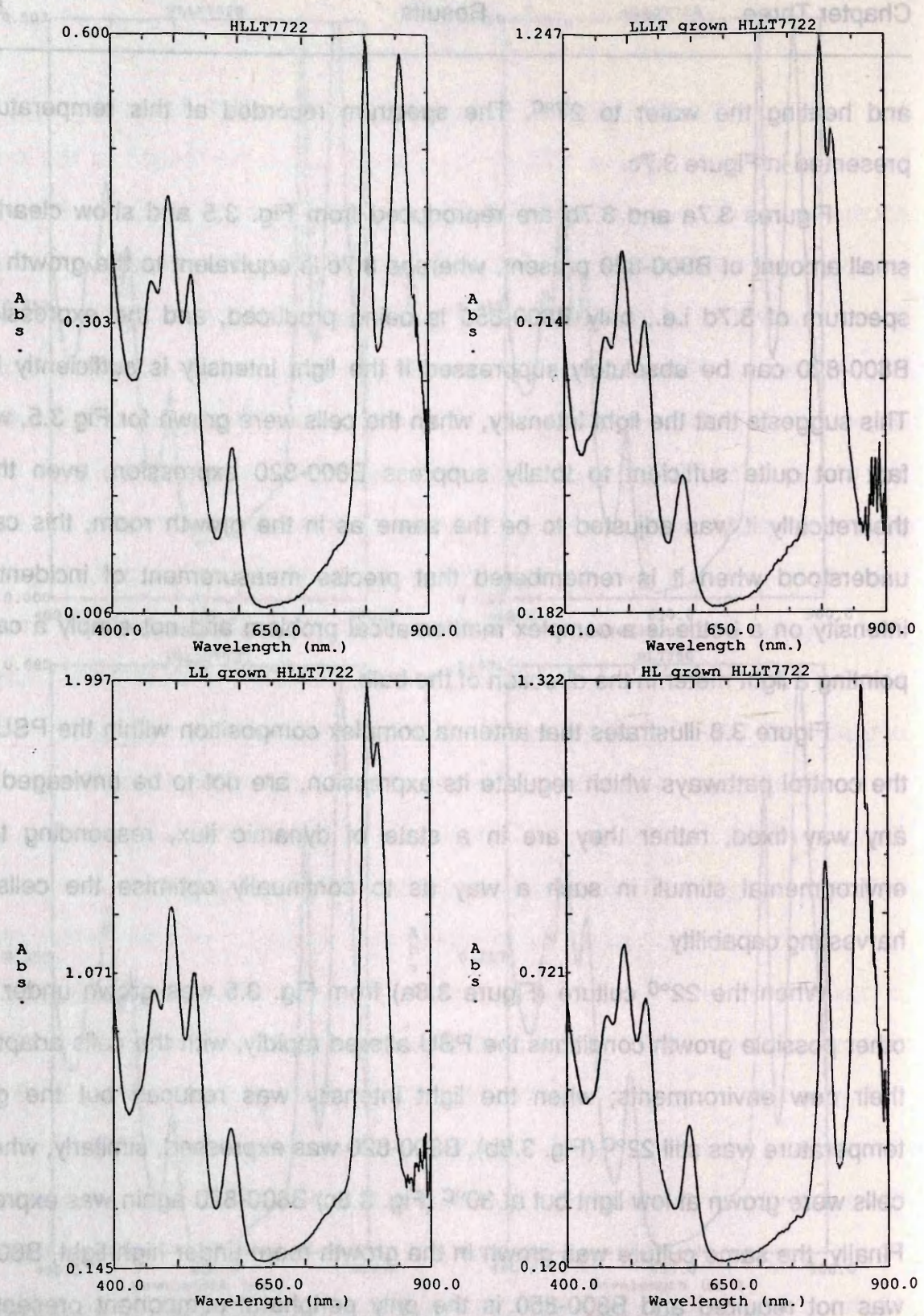



**Figure 3.8:** Experiment to demonstrate the continual turnover of the peripheral antenna in response to the incident light intensity. The top left spectrum is reproduced from Fig 3.5 (the 22°C culture), in which the peripheral antenna is mostly B800-850 with a little B800-820. The culture was grown under three different growth regimes: reduced light at 22°C (top right), reduced light at 30°C (bottom left) and high light at 30°C (bottom right). As can be easily seen the cells have adapted to each regime and the peripheral antenna complement is such that light-harvesting is optimised (as distinct from maximised) in each case.

## 3.3 Effect of light intensity and temperature on the photosynthetic unit of *Rps. acidophila* strain 7050.

This strain is known to vary both its antenna and carotenoid complement in response to changes in light intensity (Heinemeyer and Schmidt, 1983). As yet there have been no reports that either are affected by temperature, however, in view of the interesting results obtained in the last section a thorough characterisation was necessary.

The spectra obtained when this strain is grown at progressively lower light intensities are presented in Figure 3.9. This figure shows that at around 110µmols/s/m² the carotenoid composition has started to alter, from the orange, high light, rhodopin containing state to the purple, low light, rhodopinal state, yet the cells are still inserting B800-850 complex into the ICM. At the next light level the absorption spectrum in the carotenoid region has stabilised and is characteristic of the low light state in which the culture is purple, furthermore the antenna complex composition has also changed, it is apparent that the level of B800-850 present is less and B800-820 expression has started. Once the light intensity has decreased to 20µmols/s/m² the switching of the peripheral antenna is almost complete, there remains a slight shoulder at 850nm but this is lost at lower light intensities as the peripheral component becomes homogeneous. In actual fact the B800-820 complex in 7050 has its red absorption maxima nearer to 830nm than 820nm but to avoid confusion and for continuity with strain 7750 this nomenclature is maintained.

The complementary Deriphat-PAGE gel for these chromatophores is given in Figure 3.10, the change in the carotenoid composition is easily seen, the predominately rhodopin containing, orange coloured chromatophores in the 110µmols/s/m² well are replaced by the predominately rhodopinal, purple coloured chromatophores in the 40µmols/s/m² well. Moreover, the bands from the high light and 4µmol/s/m² wells were excised from the Deriphat-PAGE gel and the resultant spectra are presented in Figure 3.11. They show clearly that the change in the composition of the PSU is due exclusively to peripheral antenna switching, in the

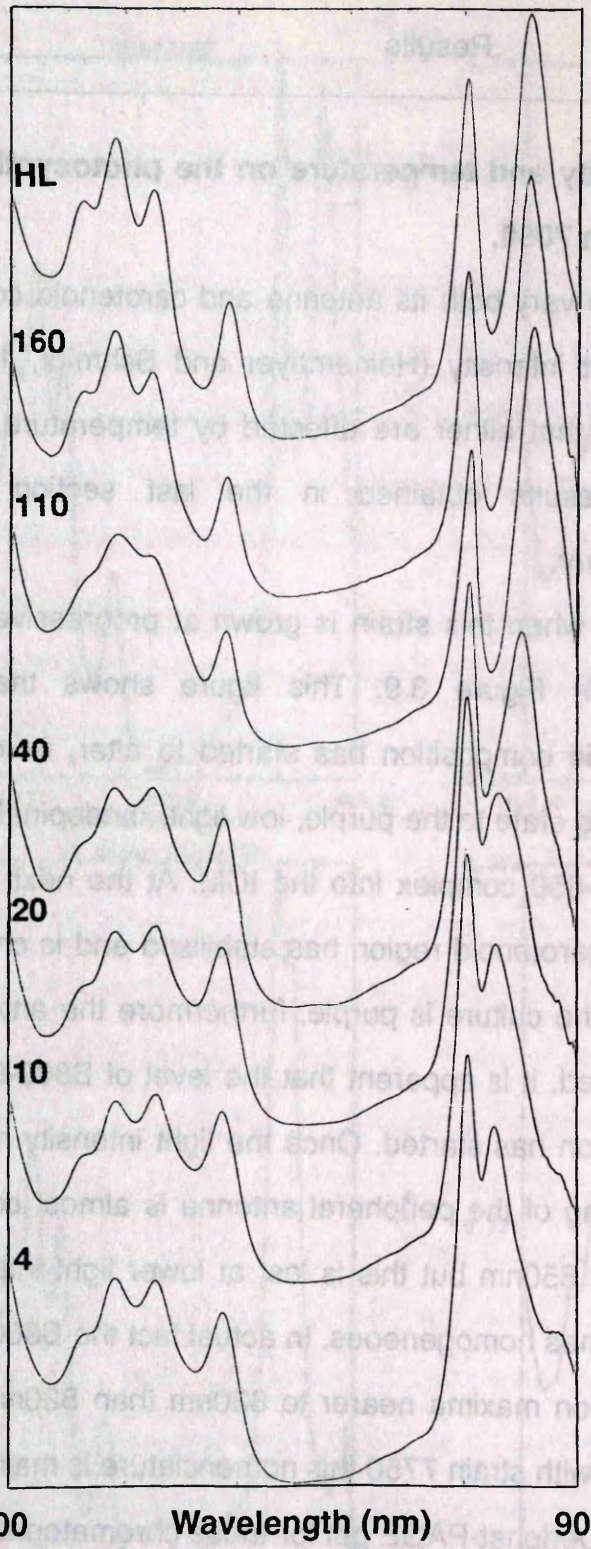



**Figure 3.9:** Spectra obtained when *Rps. acidophila* 7050 is grown at progressively lower light intensities. The incorporation of rhodopinal into the B800-850 antenna before this complex is replaced by B800-820 can be seen in the 110 and 40μmol/s/m² spectra respectively. This figure is equivalent to Fig. 3.1, which shows the results of the same regime for strain 7750.

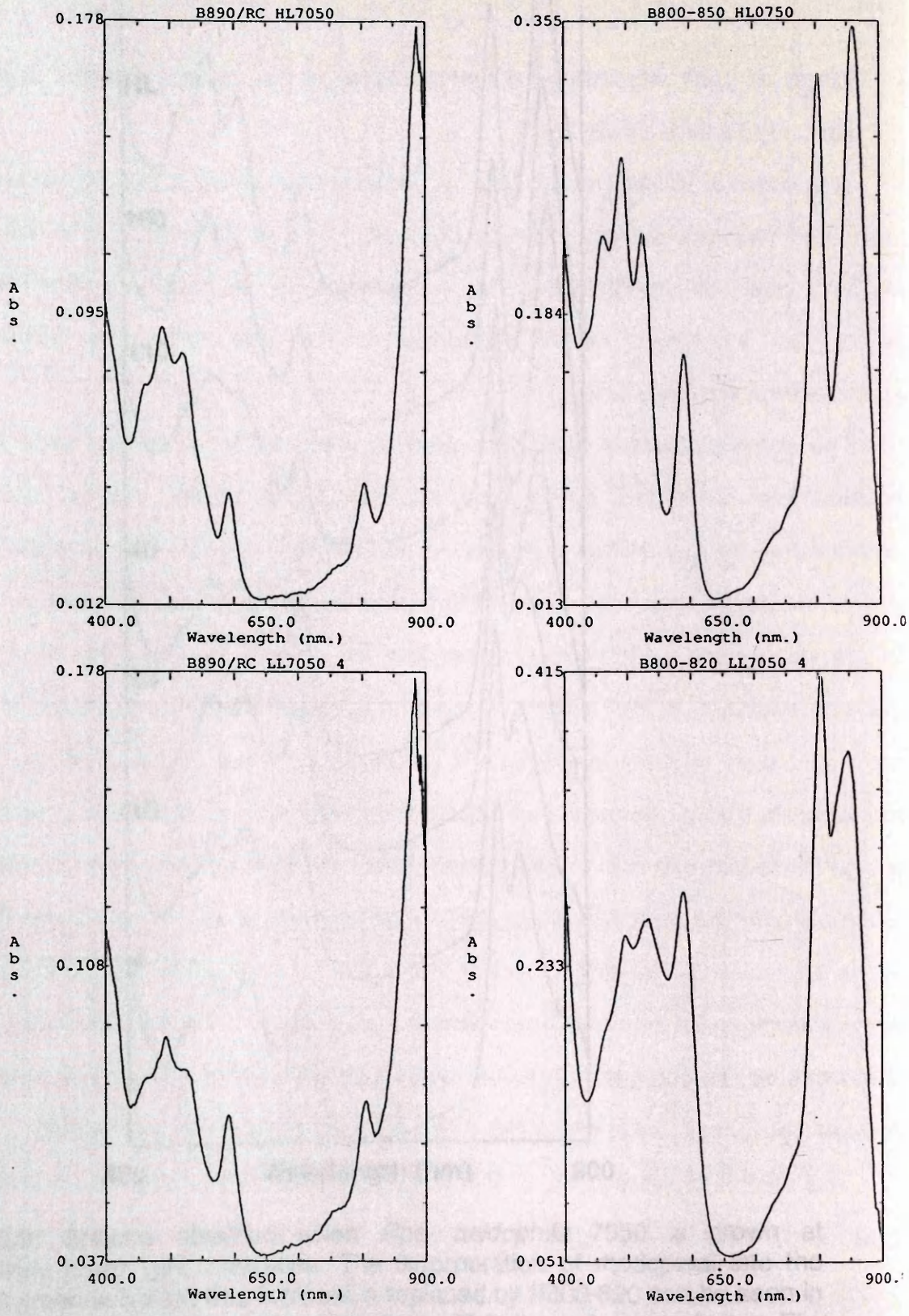



**Figure 3.11:** Spectra obtained from excised bands of high light (top row) and 4µmols/s/m² (bottom row) chromatophores from Figure 3.9. The left-hand spectra are the B890/RC conjugates and the right-hand spectra are the peripheral antenna: it can readily be seen that the carotenoid composition of each antenna type, in addition to the peripheral complex, is different. Compare with Figure 3.4.

same manner as was demonstrated for 7750 in Fig. 3.4. In contrast to 7750 the carotenoid composition also changes: the main peripheral carotenoid changes from rhodopin to rhodopinal, moreover interestingly, the carotenoid composition of the left-hand column B890/RC conjugates has also altered. This has been characterised recently (Takaichi S., pers. comm.), as involving a switch from mainly rhodopin and spirilloxanthin to primarily lycopene. Angerhofer *et al.,* (1986), have measured the efficiency of singlet-singlet transfer from carotenoid to bacteriochlorophyll in isolated antenna complexes from *Rps. acidophila* 7050. The average values obtained were 50-55% for transfer from rhodopin to Bchl in the B800-850 complex and 70-75% for transfer from rhodopinal in the B800-820 complex. Thus these values suggest the reason that this change in the carotenoid has evolved is to maximise the light harvesting potential at lower light.

To investigate the effect of temperature, if any, on this strain the cells were grown at high light but with gradually reducing temperature and the resultant spectra shown in Figure 3.12. It is apparent that neither the antenna nor the carotenoid complement have changed in this experiment. In order to determine, though, if a regulatory situation analogous to 7750 was operating i.e., high light intensity overruling any temperature induced expression, the cells were grown as before except that the light intensity was reduced by approximately one third and the resulting spectra series presented in Figure 3.13. As expected the culture at 30°C contained rhodopinal i.e., was purple in colour, and displayed an 'intermediate' NIR spectrum in that the culture contained both B800-850 and B800-820. Upon reducing the temperature no predominant increase in B800-820 expression is observed although there is a great deal of variability in the spectra.

To summarise briefly the results for this species: 7050 produces different carotenoids and peripheral antenna in response to lower light intensity but neither is affected by changes in temperature.

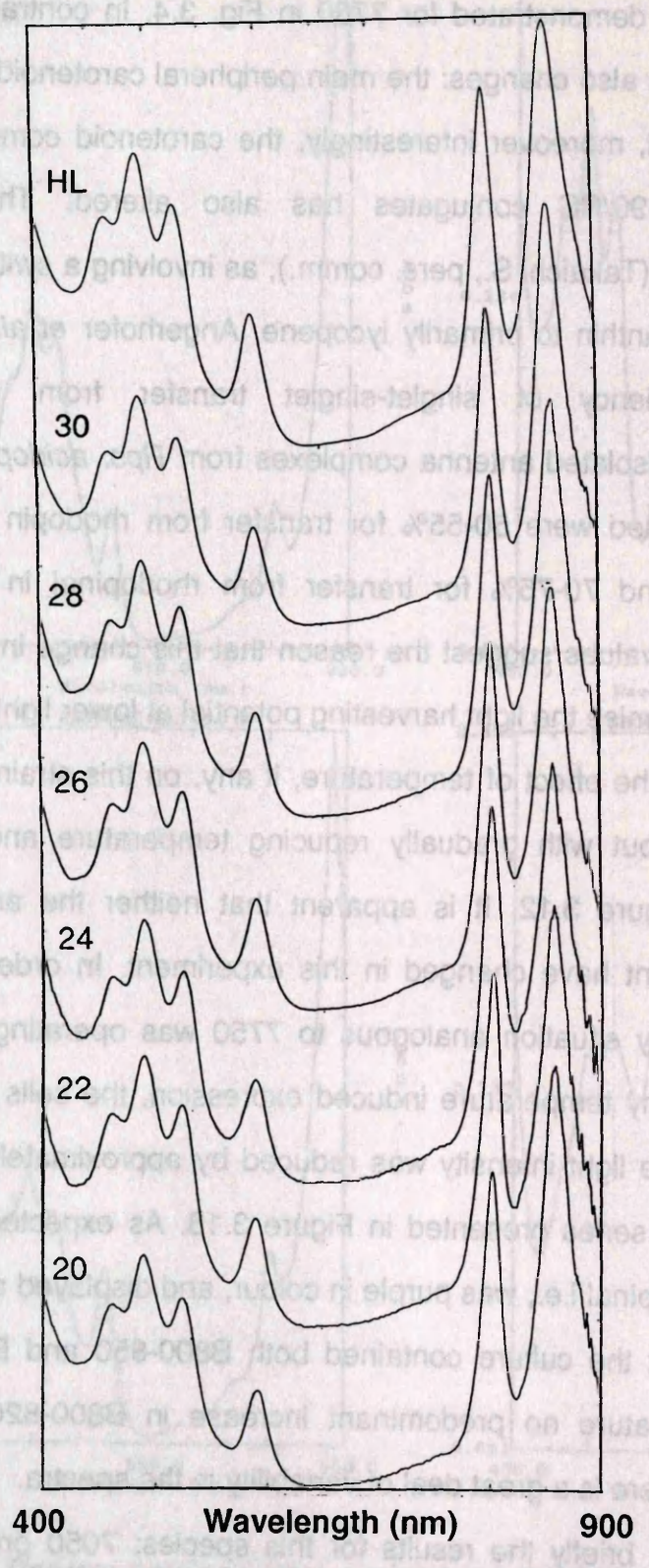



**Figure 3.12:** Spectra series obtained when *Rps. acidophila* 7050 is grown at progressively lower temperatures with high light intensity (approx. 160µmols/s/m²). This figure is comparable to Fig. 3.5, which shows the results of the same regime for strain 7750, although in this strain there is no evidence for temperature induced B800-820.

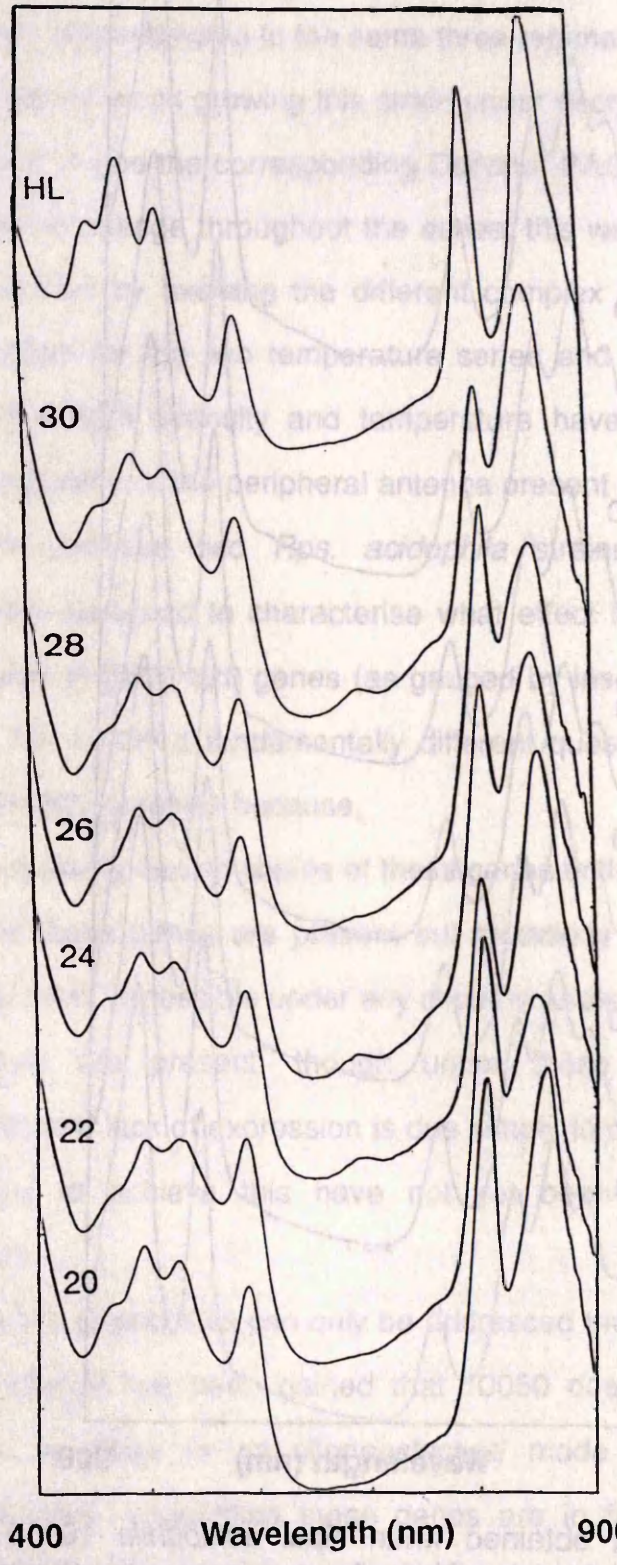



**Figure 3.13:** Spectra series obtained when *Rps. acidophila* 7050 is grown at progressively lower temperatures in conjunction with reduced light intensity (approx. 50µmols/s/m²). This figure is comparable to Fig. 3.6, which shows the results of the same regime for strain 7750. With strain 7050 there is no gradual insertion of B800-820 complex as the temperature decreases.

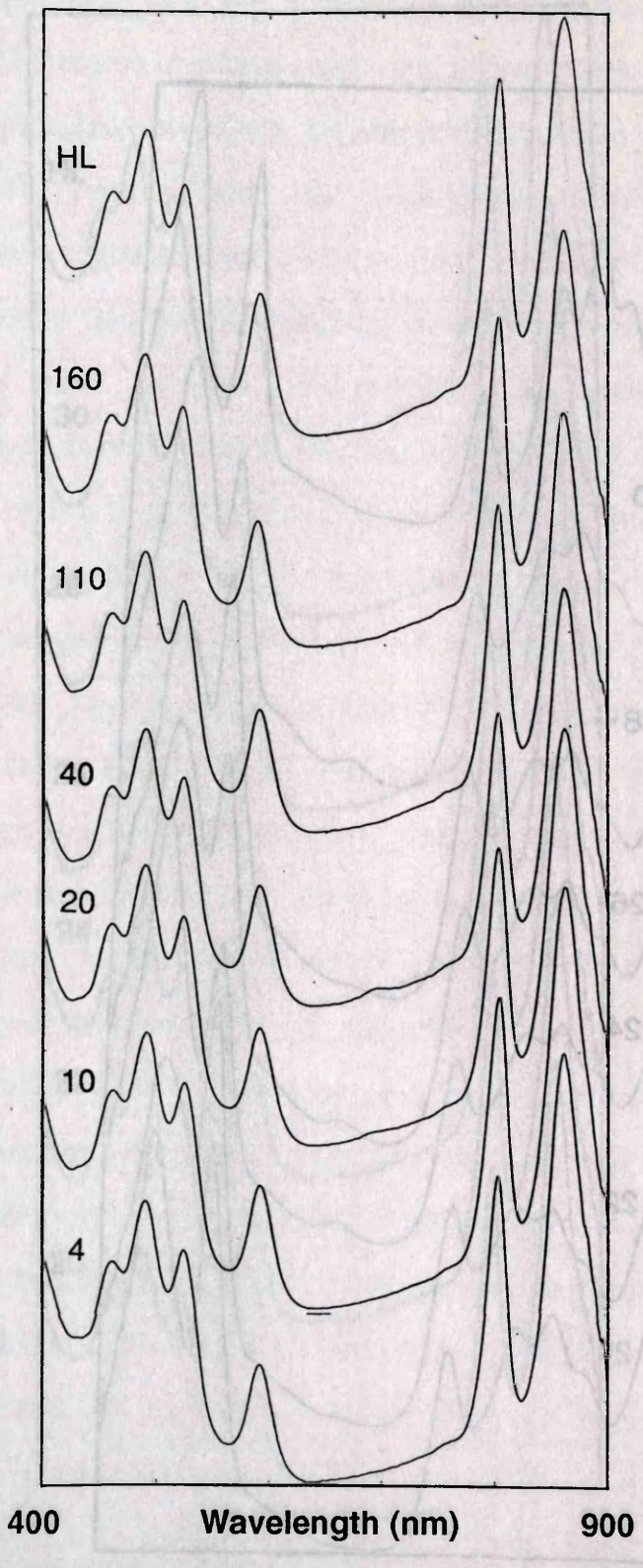



**Figure 3.14:** Spectra obtained when *Rps. acidophila* 10050 is grown at progressively lower light intensities. As previously, to obtain the spectra chromatophores from this strain were solubilised using a detergent:Bchl ratio of 35:1. This figure is equivalent to Fig. 3.1 (7750) and 3.9 (7050), however, unlike the other two strains there is no production of the B800-820 complex under these conditions.

## 3.4 Effect of light intensity and temperature on the photosynthetic unit of *Rps. acidophila* strain 10050.

This strain was subjected to the same three regimes as previously described. The spectra obtained when growing this strain under decreasing light intensities are given in Figure 3.14 and the corresponding Deriphat-PAGE gel in Figure 3.15. The spectra showed no change throughout the series, this was confirmed by recording the spectra obtained by excising the different complex bands from the gel. This result was identical for the two temperature series and therefore, the conclusion was reached that light intensity and temperature have no effect on either the carotenoid composition or the peripheral antenna present in this strain.

With the previous two *Rps. acidophila* strains, 7750 and 7050, the experiments were designed to characterise what effect light and temperature had on the expression of B800-820 genes (as gauged by insertion of these complexes into the ICM). For 10050 a fundamentally different question arises, namely is the lack of any B800-820 complex because,

(1) this strain simply has no copies of these genes in the genome.

(2) copies of these genes are present but mutations have occurred that have rendered them impossible under any circumstances to express.

(3) the genes are present, though under these conditions permanently repressed and lack of expression is due simply to the fact that the conditions necessary to achieve this have not yet been found under laboratory conditions.

The first two possibilities can only be addressed through molecular biological techniques. Evidence has been gained that 10050 does have sequences in its genome which hybridise to an oligonucleotide made from a B800-820 DNA consensus sequence, suggesting these genes are in fact present (S.J. Barrett, unpublished results), Attention switched, therefore, to asking what other environmental parameters might influence B800-820 expression. *Rps. acidophila,* which grows optimally at pH 5.5, is the member of the Rhodospirillaceae most

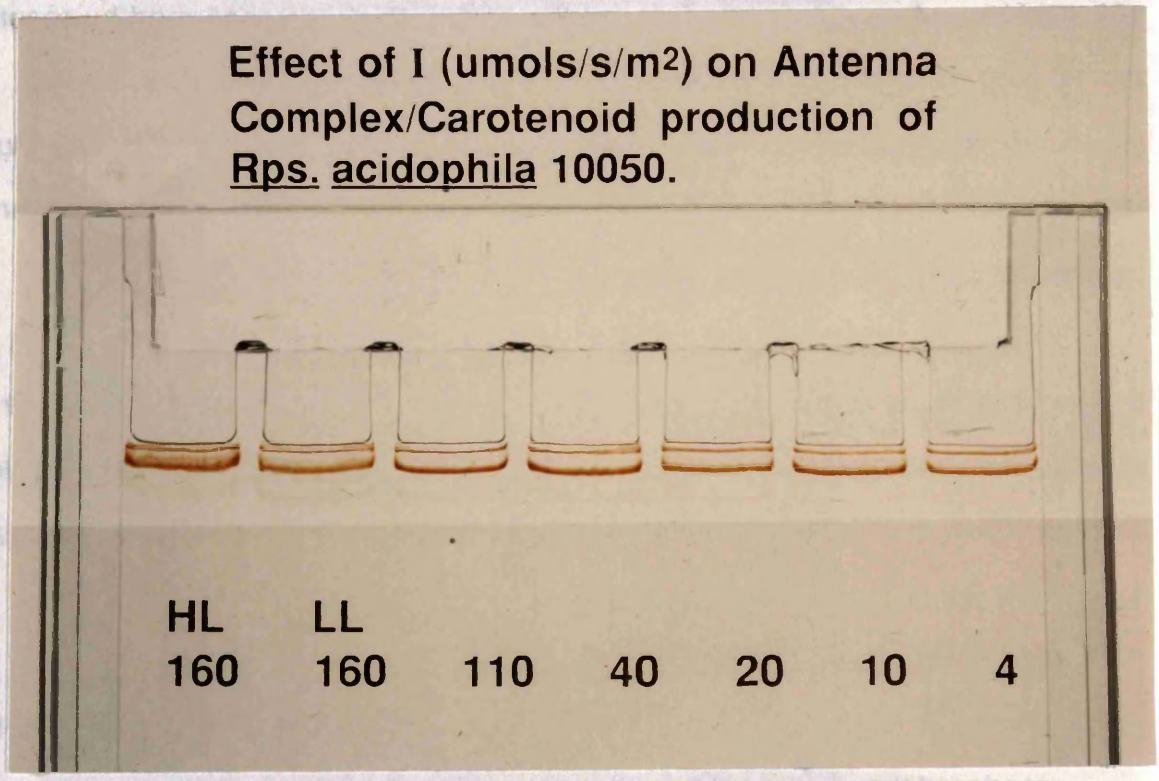



**Figure 3.15:** Deriphat-PAGE gel of *Rps. acidophila* 10050 solubilised chromatophores grown at progressively lower (from left to right) light intensities. The extreme left well contains chromatophores from cells cultured at high light. As previously the chromatophores were solubilised at a detergent:Bchl ratio of 35:1, and no evidence was attained from the excised bands that either the carotenoid composition or the antenna complement altered under this growth regime.

frequently found around the edges of peat bogs, these habitats are such that their acidic nature is not sufficient to prevent rapid production and decomposition of organic matter, which is then available for use as a carbon source. These 3 to 5 carbon compounds have different redox potentials and when assimilated and metabolised are thought to alter the redox balance i.e., the NADH/$NAD^+$-level of the cell. This ratio is also dependent on the energy charge (Gest, 1972) which is in turn dependent on the ability of the PSU to harvest the available photons. Thus there is (possibly) a direct correlation between the available carbon source in the media and the type of peripheral antenna complex present, this hypothesis received support when Heinemeyer and Schmidt, (1983) showed that *Rps. acidophila* 7050 altered both its antenna complement and its carotenoid composition when cultured on medium containing different carbon sources. Accordingly it appears logical to propose that expression of 10050 B800-820 genes might be possible if compounds other than succinate are used as the carbon source

To test this Pfennig's media was prepared with pyruvate and with malate that contained an equivalent amount of carbon as the usual succinate medium containing form (0.2mol/l), these two substrates were chosen for the following reasons:

(1) Both are able to be assimilated and metabolised by 10050.

(2) Both have opposite redox potentials ($E_o'$), outlined in Table 3.1.

**Table 3.1. Standard redox half reactions for the carbon sources used to make Pfennigs media.**

| Half reaction | $E_o'$ (V) |
|---|---|
| succinate $\rightarrow$ fumarate + $2H^+ + 2e^-$ | -0.03 |
| pyruvate + $2H^+ + 2e^- \rightarrow$ lactate | -0.19 |
| malate $\rightarrow$ oxaloacetate+ $2H^+ + 2e^-$ | 0.17 |

**$E_o'$ = standard oxidation-reduction potential (pH 7.0, 25°C).**

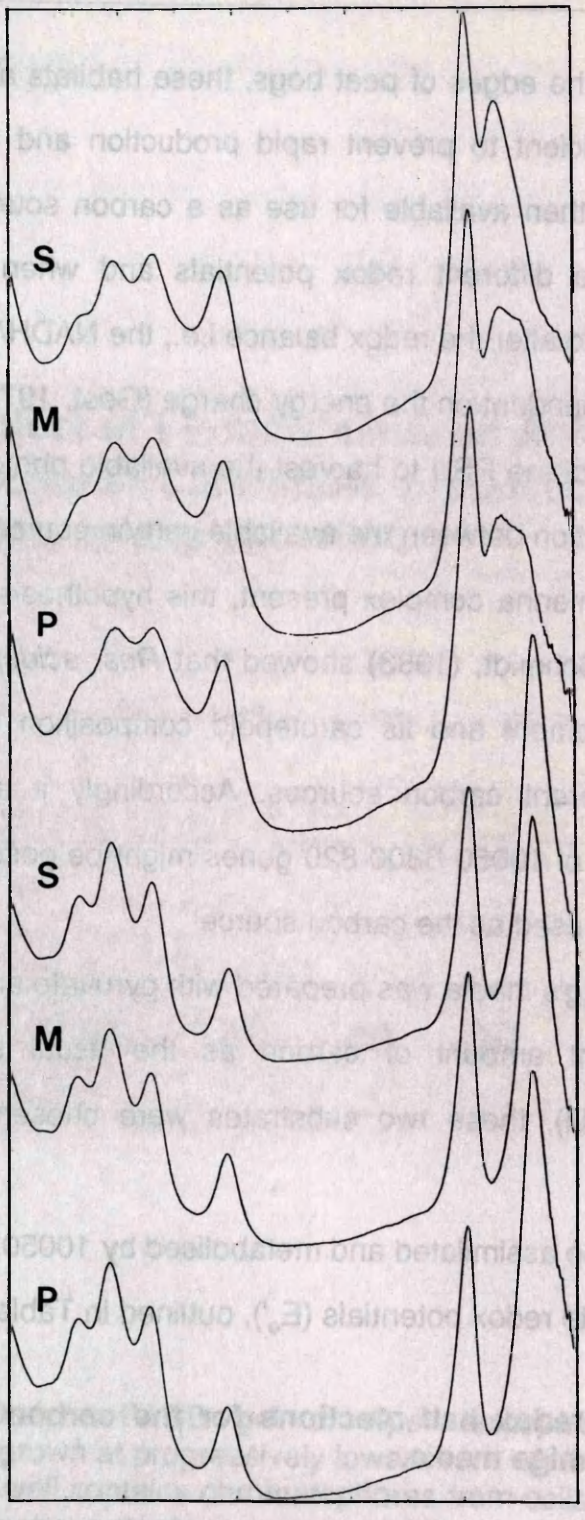



**Figure 3.16:** Spectra obtained when *Rps. acidophila* 10050 and *Rps. acidophila* 7050 are grown at low light intensity (10µmols/s/m²) using media containing different carbon sources (0.2mol/litre carbon); S, succinate; P, pyruvate; M, malate. It is apparent that the carbon source affects the peripheral antenna of strain 7050 but has no effect in this experiment with strain 10050.

These media were then used to culture both 10050 and 7050 at an light intensity of 10μmols/s/m², the rationale being that if the metabolism of the carbon source has resulted in a low energy charge within the cell, this would be enhanced by the low light: perhaps to the extent that B800-820 expression is initiated in an attempt by the cell to redress the deficiency in the energy charge through an increased light harvesting efficiency. The resultant spectra are presented in Figure 3.16.

The spectra reveal that 10050 grown on either malate or pyruvate (or succinate to act as a control) does not induce B800-820 complex expression. *Rps. acidophila* 7050 was also cultured on these three carbon sources, although in this case there were differences induced in the antenna complement. Succinate, as expected, produced only the B800-820 complex, whereas both malate and pyruvate induced also a small amount of B800-850 expression.

Before a possible conjectural explanation for these phenomena is forwarded it is neccessary to consider the change in free energy for the carbon source in conjunction with the production/consumption of reducing equivalents, as the first step in the metabolism of this compound. The standard free energy change values in this regard are given in Table 3.2.

**Table 3.2. Standard free energy change associated with the carbon sources oxidation/reduction by $NAD^+$/NADH.**

| Full reaction with $NAD^+$/NADH couple. | $\Delta G^{\circ\prime}$ (kJ/mol) |
|---|---|
| succinate + $NAD^+ \rightarrow$ fumarate + NADH + $H^+$ | +67.5 |
| pyruvate + NADH + $H^+ \rightarrow$ lactate + $NAD^+$ | -25.1 |
| malate + $NAD^+ \rightarrow$ oxaloacetate + NADH + $H^+$ | +28.9 |

**where $\Delta G^{\circ\prime} = -nF\Delta E_o'$**
**n = no. of $e^-$ transferred from reductant to oxidant.**
**F = Faraday constant (96.49 kJ/mol).**

These values must be interpreted with caution as standard conditions do not apply *in vivo* (growth temperature was 30°C not 25°C and *Rps. acidophila* media is pH 5.2 therefore the cytosol is unlikely to be pH 7.0), however, some inferences are able to be drawn particularly with regard to the qualitative differences between succinate and malate. The enzyme that oxidises succinate, Succinate Dehydrogenase (SDH), with concomitant production of NADH is located in the photosynthetic membrane (see Figure 1.2), and thus associated with ATP production. Growth on malate probably results in either induction of an equivalent Malate Dehydrogenase, or SDH has a broad specificity that enables it to metabolise malate. In either event a direct comparison between malate and succinate would appear to be valid, however this is not the case for pyruvate which as is evident from Table 3.2 is reduced by NADH and results in the production of lactate, which (by analogy with *E. coli*) is a metabolic dead end. Therefore, this compound, which has a pivotal role in the cell's metabolism probably enters these pathways by either decarboxylation to form Acetyl-CoA and hence, e.g., lipid anabolism or carboxylation as the first step in gluconeogenesis.

Taking all of this into account a speculative hypothesis for the spectra obtained in Fig 3.16 can now be forwarded. Firstly, for *Rps. acidophila* 7050, the large positive $\Delta G^{o'}$ value for succinate compared to malate suggests that the production of 1mol of NADH from succinate is much more of a burden for the cell than when malate is the carbon source, therefore, when the cell is growing at low light on succinate it must ensure the energy charge is as close to 1 as possible. To ensure this the cell produces only B800-820, thereby light harvesting is maximised. With malate as the carbon source the equivalent burden is considerably less and as such the necessity to maximise the light harvesting capability is reduced to such an extent that the B800-820 complex (efficient light-harvester) has been diluted with B800-850 (less efficient). Empirically, this latter case appears true when pyruvate is the carbon source, however, for the reasons outlined above this compound can not be directly compared with succinate or malate.

The initial aim of this experiment was to attempt to induce B800-820 expression in *Rps. acidophila* 10050. If the rationale outlined in this section and the explanation for the 7050 spectra just given, is an accurate account of the relationship between carbon source and antenna gene expression, then it is apparent that neither malate nor pyruvate are able to stress the cell sufficiently to trigger B800-820 expression. Therefore the conclusion must be drawn that in 10050 B800-820 expression is unlikely, however it might be still possible if a carbon source was available that fulfilled the following conditions (1) it had a $E_o'$ value <-0.03, (2) was assimilated by 10050 and also, (3) was oxidised by $NAD^+$ and resulted in a product that was easily further metabolised.

## 3.5 **Conclusions.**

Evidence has been presented that two of the three strains of *Rps. acidophila* studied are able to adapt to different environmental conditions by changing either its peripheral antenna complex carotenoid composition and/or complement. These can be summarised as follows:

Strain 7750 is able to produce B800-820 in response to either low light or low temperature, however, the light regulation appears to take precedence over temperature regulation i.e., growth at high light but low temperature will result in the peripheral antenna consisting solely of B800-850. No evidence was found that the carotenoids in this strain are affected by either parameter.

Strain 7050 is able to produce B800-820 and rhodopinal only in response to low light, furthermore, this carotenoid change precedes the switch of peripheral antenna and is evident also in the fixed antenna. Temperature was found to have no effect on either the peripheral antenna complement or the carotenoid composition, however, the carbon source on which the cells were grown did have an effect.

Strain 10050 was unable to alter either its peripheral antenna complement or carotenoid composition in response to light, temperature or the carbon source on which the cells were grown.

# Chapter Four

## HPLC analysis of *Rps. acidophila* carotenoids.

## 4.1 Introduction.

The carotenoid composition present in *Rps. acidophila* strains 7050 (Heinemeyer and Schmidt, 1983) and 7750 (Clayton, 1978) has been characterised previously. For strain 10050 the composition is known to be similar (R. J. Cogdell, pers. comm.), although nothing as yet has been published. All of the strains investigated synthesise carotenoids of the 'normal spirilloxanthin series' (Fig. 1.6), except that rhodopin rather than spirilloxanthin is accumulated as the main product. Moreover, the occurrence of a high content of carotenoid glucosides is known to be characteristic of this organism (Schmidt, 1971).

The aims of this chapter were to analyse in detail and compare: (1) the carotenoid composition of the three strains growing under a defined high light intensity, and (2) the changes induced by low light in 7050 and 7750 (if any), using reversed phase HPLC. In general terms the order of elution with reversed phase chromatography depends on the hydrophobicity of a compound. The greater the surface area of the apolar region of the molecule the greater will be the interaction with the stationary phase. Polar hydrophilic compounds, therefore, will elute before non-polar hydrocarbons since the former are more weakly retained.

Four main methods are used to identify carotenoids; chromatographic behaviour, UV-visible light spectroscopy, mass spectrometry (m.s.) and nuclear magnetic resonance (NMR). Identification of the carotenoids present in *Rps. acidophila* by all four methods has been accomplished by Connor, (1991), using an Acetonitrile / Water / Ethyl acetate solvent system in conjunction with an $C_{18}$-ODS column. The carotenoids presented in the forthcoming chromatograms were produced on the same system, and identified using a combination of the retention order established by Connor, and absorption spectra data. As no m.s. or NMR was performed, identification of the carotenoids must be regarded as not categorical.

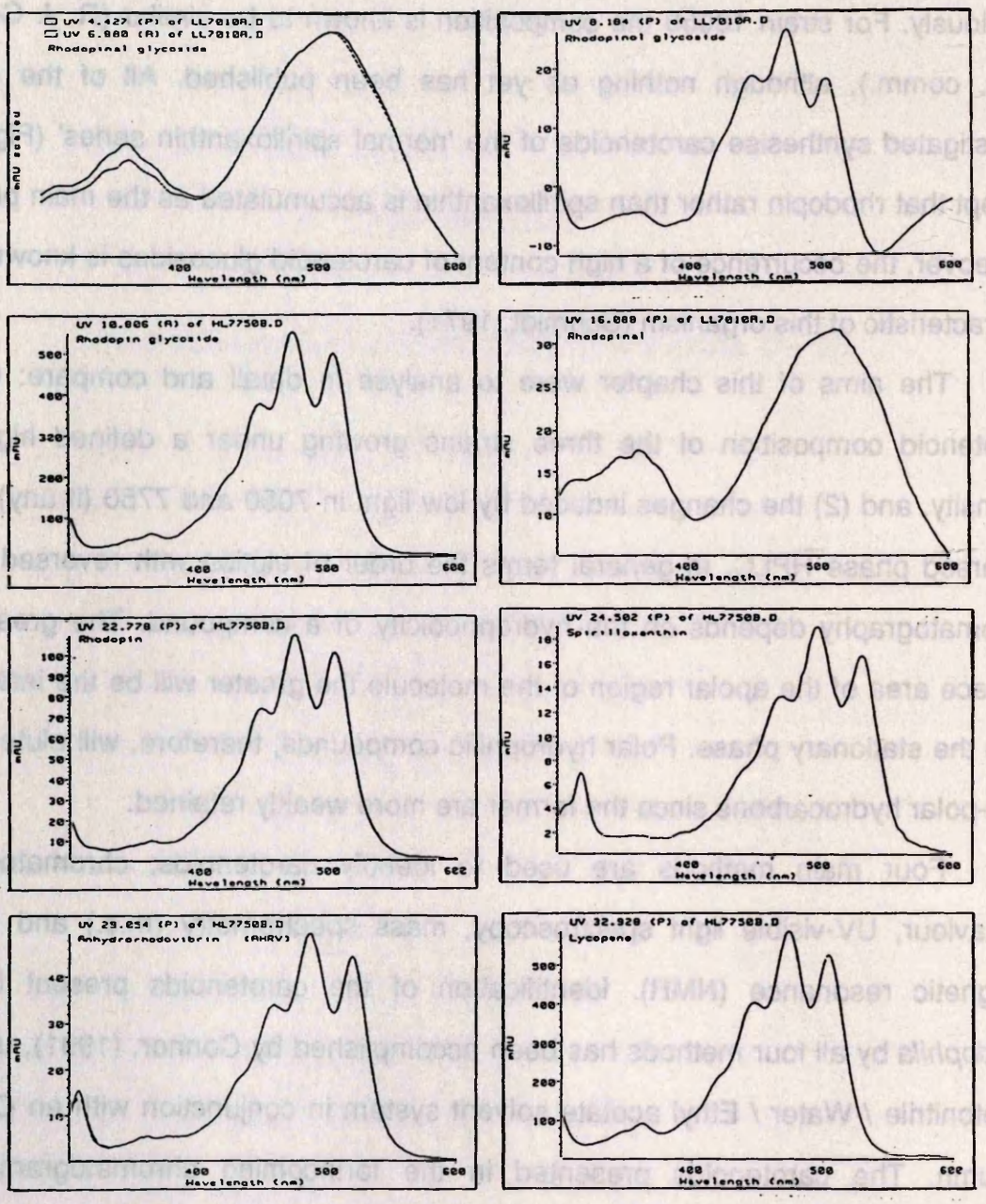



**Figure 4.1:** Sample spectra of each carotenoid obtained from either high light grown *Rps. acidophila* 7750, or low light grown 7050, along with their putative identification. Because rhodopinal glycoside eluted over such a wide band (see Figure 4.6) spectra were recorded at 4.4 and 6.9min and normalised at 510nm. The former appears to be the *cis*-isomer, as such they overlie each other above 400nm but not below this wavelength.

## 4.2 Identification and quantitation of *Rps. acidophila* carotenoids.

The following chromatograms reveal that the three *Rps. acidophila* strains produce in total eight main carotenoids. A specimen absorption spectra for each carotenoid is presented in Figure 4.1. As mentioned above the carotenoids present in this species are well characterised: with regard to reversed phase HPLC the study by Connor, (1991) was especially relevant. A summary of this data is given in Table 4.1. Although it was also possible to detect but not identify trace amounts (<1% of total carotenoid) of other carotenoids, probably intermediates such as e.g. lycopen-2-ol, rhodopinol or OH-spirilloxanthin; they have been omitted from the analysis.

**Table 4.1. Summary of *Rps. acidophila* carotenoid data (reproduced from Connor, 1991).**

| Carotenoid | Ret. time (min) | Strain | $\lambda_{max}$ (nm) | N |
|---|---|---|---|---|
| **Rhodopinal glucoside** | **11.7** | **7050** | **510** | **11** |
| **Rhodopinol glucoside** | **13.1** | **7050** | **473** | **11** |
| **Rhodopin glucoside** | **15.7** | **7750** | **473** | **11** |
| **Rhodopinal** | **18.0** | **7050** | **510** | **11** |
| **Rhodopin** | **26.7** | **7750** | **473** | **11** |
| **Spirilloxanthin** | **27.5** | **7750** | **497** | **13** |
| **Anhydrorhodovibrin** | **31.7** | **7750** | **484** | **12** |
| **Lycopene** | **35.6** | **7750** | **473** | **11** |

**N denotes the number of conjugated double bonds in the molecule. The $\lambda_{max}$ value was recorded in the 10% water:acetonitrile/ethyl acetate solvent gradient used for this analysis.**

The retention times given have no meaning in themselves but serve only to illustrate the order in which the carotenoids elute off the column. Retention times and respective $\lambda_{max}$ values are given in Table 4.2 for the eight main carotenoids that were produced by *Rps. acidophila* in the chromatograms to be presented in this chapter.

**Table 4.2. Retention times and $\lambda_{max}$ wavelengths obtained from all eight *Rps. acidophila* carotenoids identified in this study.**

| Car. ret. time (min) | $\lambda_{max}$ (nm). |
|---|---|
| 6.9 | 510 |
| 8.2 | 473 |
| 10.8 | 473 |
| 16.1 | 510 |
| 22.8 | 473 |
| 24.5 | 497 |
| 29.1 | 485 |
| 32.9 | 473 |

**The $\lambda_{max}$ value was recorded in the 10% water:acetonitrile/ethyl acetate solvent gradient used for this analysis.**

The energies of the excited singlet ($^1B_{2u}^*$) [and most probably also the triplet] states of carotenoids depend upon the degree of $\pi$-electron conjugation. In general, the more double bonds in the polyene chain the lower the spectroscopic energies of both states (Mathis and Kleo, 1973) and light of longer wavelength will be absorped. For a carotenoid such as phytoene in which N=3, $\lambda_{max}$ in methanol is 285nm. Most, if not all photosynthetic organisms only accumulate carotenoids containing more than seven conjugated double bonds and the $\lambda_{max}$ value will be correspondingly increased, e.g., spirilloxanthin (N=13) has its $\lambda_{max}$ in methanol at 492nm. Thus, there is a direct correlation between $\lambda_{max}$ and N.

This then allows identification of the carotenoids eluting at 24.5 and 29.0 min which have absorption maxima at 497 (N = 13) and 485nm (N = 12) respectively; from Table 4.1, there are only two carotenoids which can be isolated from *Rps. acidophila* for which N does not equal 11. Correspondingly, the carotenoid at 24.5 can be identified as spirilloxanthin and similarly anhydrorhodovibrin (AHRV) at 29.0min.

Polar groups such as hydroxyl, methoxy, keto and aldehyde groups influence retention times on reversed phase HPLC and their effect is cumulative e.g.,

retention time becomes shorter as the number of hydroxyl groups increases or the polarity due to two methoxy groups roughly corresponds to one hydroxyl. Thus, provided the types of carotenoid in any given sample are known (as is the case from Table 4.1), the kinds and numbers of the polar groups and hence the identity of the carotenoid, can be inferred from the retention time.

Comparison of those carotenoids with $\lambda_{max}$ at 473nm, in conjunction with retention times given in Table 4.1, allows the carotenoid eluting at 32.9min to be identified as lycopene. This is the major precursor for most prokaryotic photosynthetic carotenoid anabolism and as such is unsubstituted, therefore, it will adsorb strongly to the stationary phase, resulting in a long retention time. Similarly the carotenoid at 22.8min is rhodopin, structurally similar to lycopene except it has a hydroxyl added at C-1 thereby increasing the molecule's polarity and decreasing the retention time. The occurrence of an unusually high content of carotenoid glucosides is characteristic of *Rps. acidophila* and in all known cases the sugar moiety forms a glycosidic linkage through the C-1 hydroxyl of the carotenoid. This attachment greatly increases the polarity of the molecule and so shortens the retention time, thus rhodopin glucoside elutes at 10.8min.

In Chapter 3 it was shown that 7050 produces rhodopinal at low light intensities. The formation of rhodopinal from rhodopin is an anaerobic process, in which the $C_{20}$-methyl group is oxidised to an aldehyde group (the intermediate rhodopinol glucoside can be isolated in its own right). As this molecule contains two hydroxyl groups it will elute before rhodopin glucoside at 8.2min. The remaining two spectra are easily identified as rhodopinal at 16.1min and its glucoside at 6.9min, due to their distinctive spectra which have a single well rounded peak with $\lambda_{max}$ at 510nm. There is however a degree of contention as to whether the Connor, (1991) identification of rhodopinol glucoside is correct: Dr. S. Takaichi (pers. comm.) has asserted that this carotenoid should elute before rhodopinal glucoside because the hydroxyl group is more polar than the aldehyde. The data from Connor, (1991) places this carotenoid after rhodopinal glucoside. Therefore, either her identification

is wrong or the anomalous retention order is a peculiarity of this particular solvent system. In either event the identification of rhodopinol glucoside is noted to be tentative.

The elution characteristics of the main light harvesting pigment, bacteriochlorophyll are illustrated in Figure 4.2. The majority of the pigment elutes in a wide band, centred on a retention time at 17.9 (for this strain 7750), between rhodopin glucoside and rhodopin. The major breakdown product, bacteriopheophytin (Bpheo) elutes at approx. 32.7, the loss of the liganded $Mg^{2+}$ from the tetrapyrrole ring causes a marked decrease in the polarity of the molecule and a blue shift in the Qx dipole absorption.

The amount of any particular carotenoid is given as a percentage of the total carotenoid, (that could be identified), present in that chromatogram. To avoid underestimating the true peak area, all carotenoid peaks were integrated from chromatograms recorded at their respective their $\lambda_{max}$ wavelength.

Unless otherwise stated each run was repeated three times i.e., two different membrane preparations one of which was run twice and the requisite peaks integrated. The integration results at 473nm from the chromatogram illustrated in Figure 4.2 are presented in Figure 4.3 and a sample calculation for this data illustrated in Table 4.3, alongside data from 484 and 497nm where necessary.

It might have been expected that as these carotenoids comprise of virtually all of the total carotenoids in the sample, the total area of carotenoid when summed would approximate 50% that of bacteriochlorophyll. In fact it is considerably greater, this has been shown to be due to the lability of Bchl under these conditions, often breakdown exceeds 20-30% of the total, whereas carotenoid recovery off the column approaches 100% (Evans, M. B., pers. comm.).

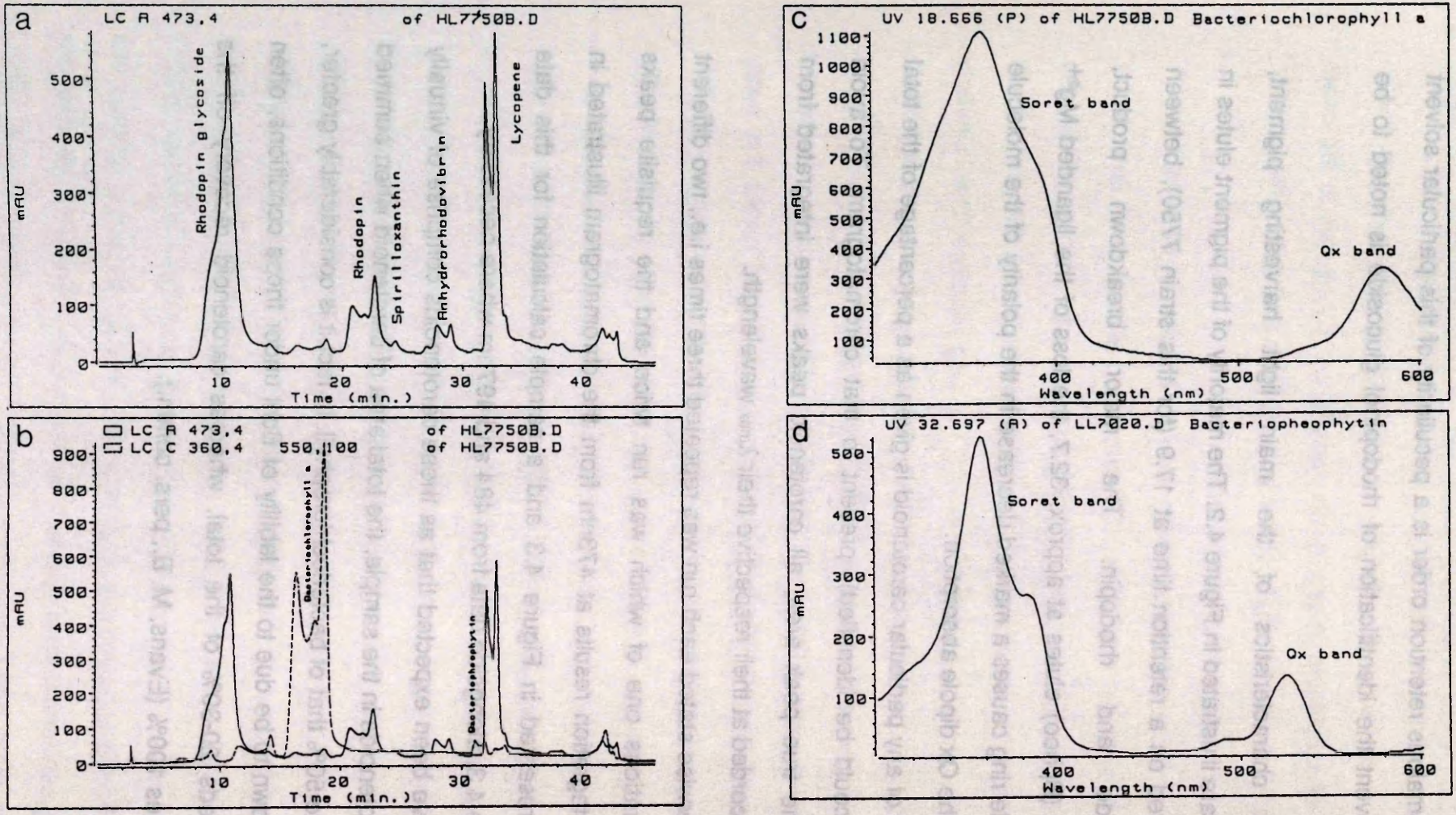
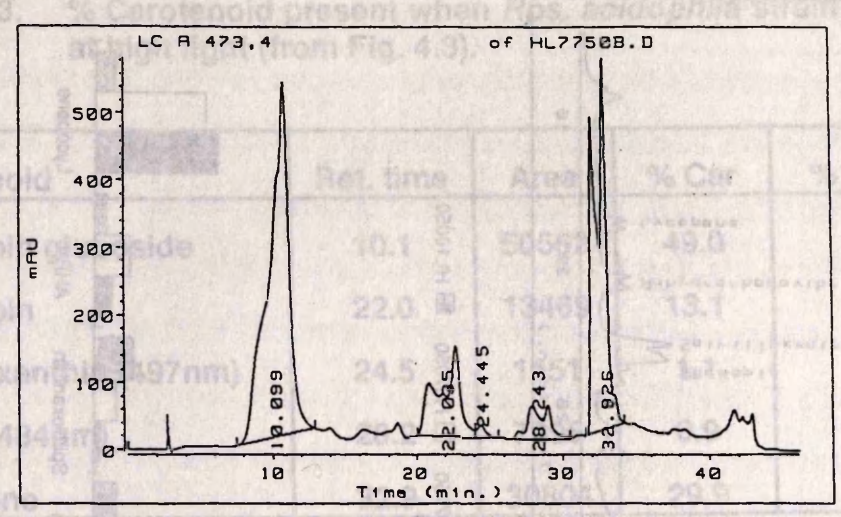
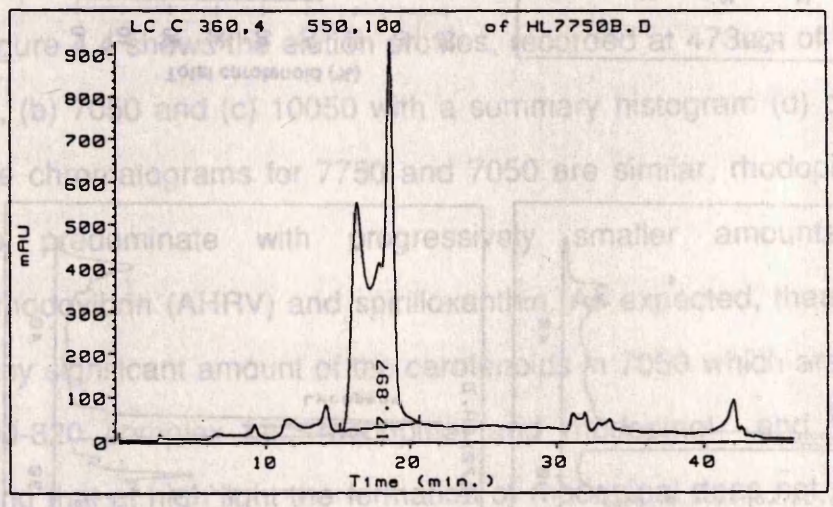



**Figure 4.2: (a)** Chromatogram (recorded at 473nm) illustrating the carotenoid composition of high light grown 7750. This was repeated **(b)** with the same sample, (recorded at 360nm) to determine when Bchl and Bpheo elute, and then superimposed on the 473nm trace. **(c)**and **(d)** are spectra of Bchl and Bpheo respectively.




LC A 473,4 of HL7750B.D
DATA:HL7750B.D

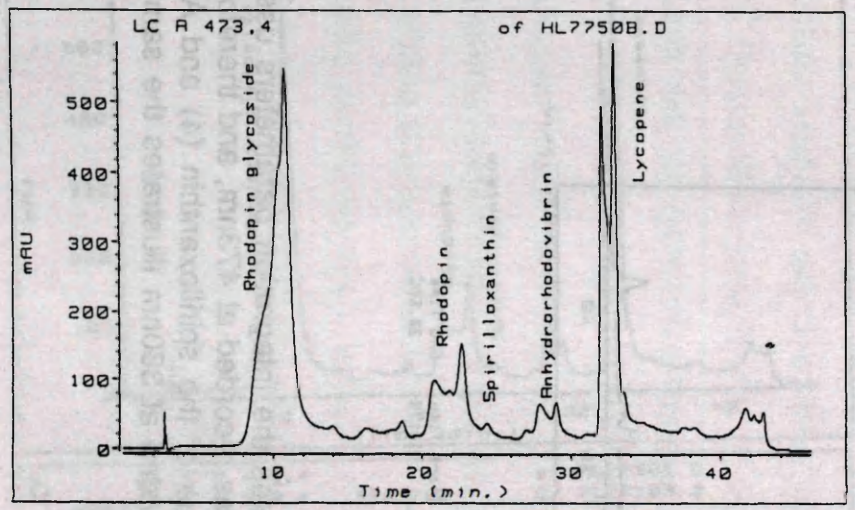
| Peak# | Ret Time | Type | Width | Area | Start Time | End Time |
|---|---|---|---|---|---|---|
| 1 | 10.099 | BB + | 0.000 | 50562 | 7.491 | 12.910 |
| 2 | 22.035 | BV + | 0.000 | 13469 | 20.025 | 24.046 |
| 3 | 24.445 | VV | 0.507 | 777.53 | 24.046 | 25.626 |
| 4 | 28.243 | PV + | 0.000 | 4860 | 26.438 | 30.481 |
| 5 | 32.926 | BBA+ | 0.000 | 30804 | 31.566 | 34.185 |

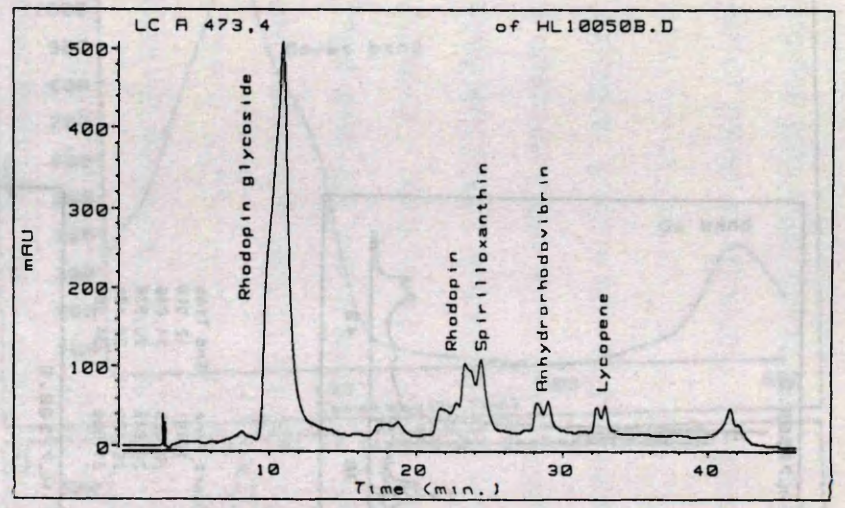


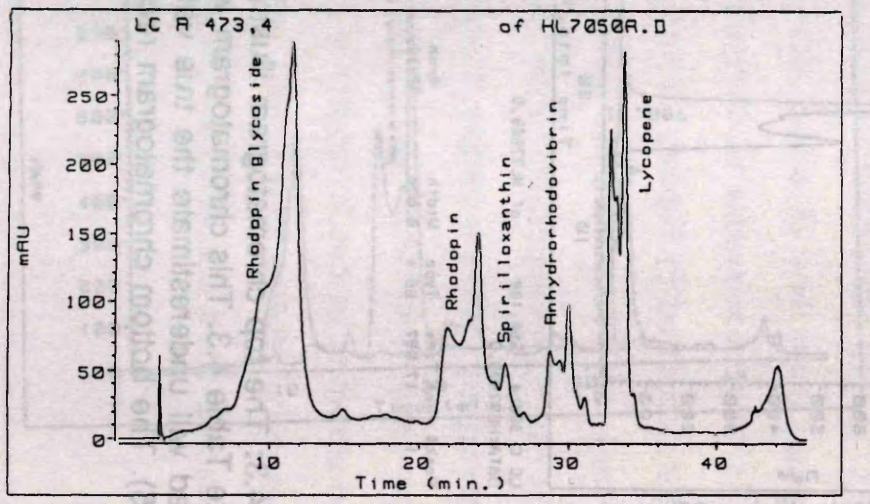

LC C 360,4 550,100 of HL7750B.D
DATA:HL7750B.D

| Peak# | Ret Time | Type | Width | Area | Start Time | End Time |
|---|---|---|---|---|---|---|
| 1 | 17.897 | BB + | 0.000 | 92211 | 15.129 | 20.665 |

**Figure 4.3:** The top chromatogram illustrates the integration parameters used to formulate Table 4.3. This chromatogram was recorded at 473nm, and therefore, if integrated will underestimate the true value of the spirilloxanthin (4) and AHRV peaks (5). The bottom chromatogram recorded at 360nm illustrates the same for Bchl.



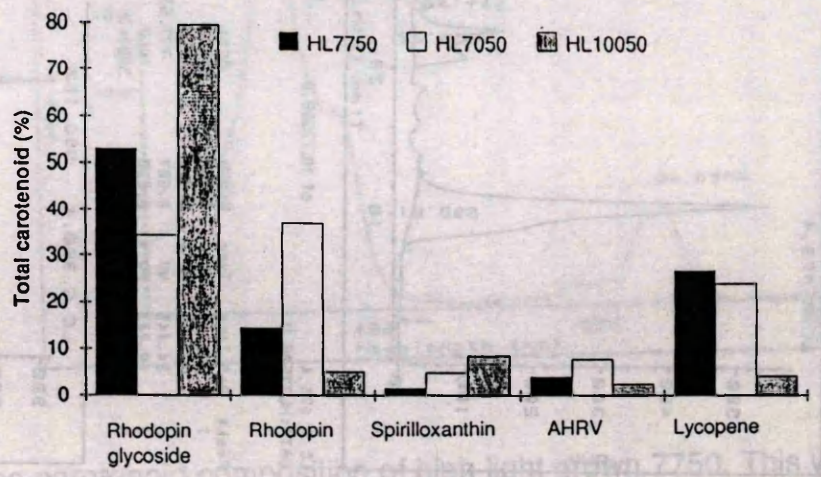




**Figure 4.4:**Chromatograms showing the elution of carotenoids from high light grown; **(a)** 7750, **(b)** 7050 and **(c)** 10050;the amounts of each carotenoid present, taken from the mean of three runs, are presented **(d)** in a histogram for ease of comparison.

**Table 4.3. % Carotenoid present when *Rps. acidophila* strain 7750 is grown at high light (from Fig. 4.3).**

| Carotenoid | Ret. time | Area | % Car | % Mean (n=3) |
|---|---|---|---|---|
| Rhodopin glucoside | 10.1 | 50562 | 49.0 | 52.9±3.4 |
| Rhodopin | 22.0 | 13469 | 13.1 | 14.6±1.1 |
| Spirilloxanthin (497nm) | 24.5 | 1151 | 1.1 | 1.6±0.3 |
| AHRV (484nm) | 28.2 | 7096 | 6.9 | 4.1±1.4 |
| Lycopene | 32.9 | 30804 | 29.9 | 26.7±2.0 |

**The total area of carotenoid recovered and identified = 103082**
**n = number of runs from which mean was taken.**

## 4.3 Carotenoid composition at high light intensity.

Figure 4.4 shows the elution profiles, recorded at 473nm of the three strains (a) 7750, (b) 7050 and (c) 10050 with a summary histogram (d) of the integration data. The chromatograms for 7750 and 7050 are similar, rhodopin glucoside and lycopene predominate with progressively smaller amounts of rhodopin, anhydrorhodovibrin (AHRV) and spirilloxanthin. As expected, there is an apparent lack of any significant amount of the carotenoids in 7050 which are associated with the B800-820 complex i.e., rhodopinal and rhodopinol and their glucosides, suggesting that at high light the formation of rhodopinal does not occur, or at least not to any substantial degree. This is in general agreement with the results presented in Chapter 3, and Figure 3.10 in particular. Previous studies (Connor, 1991; Heinemeyer and Schmidt, 1983) proport to show that, for example, rhodopinal glucoside in high light 7050 cells accounts for 21 and 38% respectively of the total carotenoid. This can be resolved with a closer examination of the absorption spectra of the cells from which the carotenoids in these studies were extracted: both show a small but pronounced degree of B800-820 complex present, therefore, it appears the cells had been grown at a high light intensity but not high

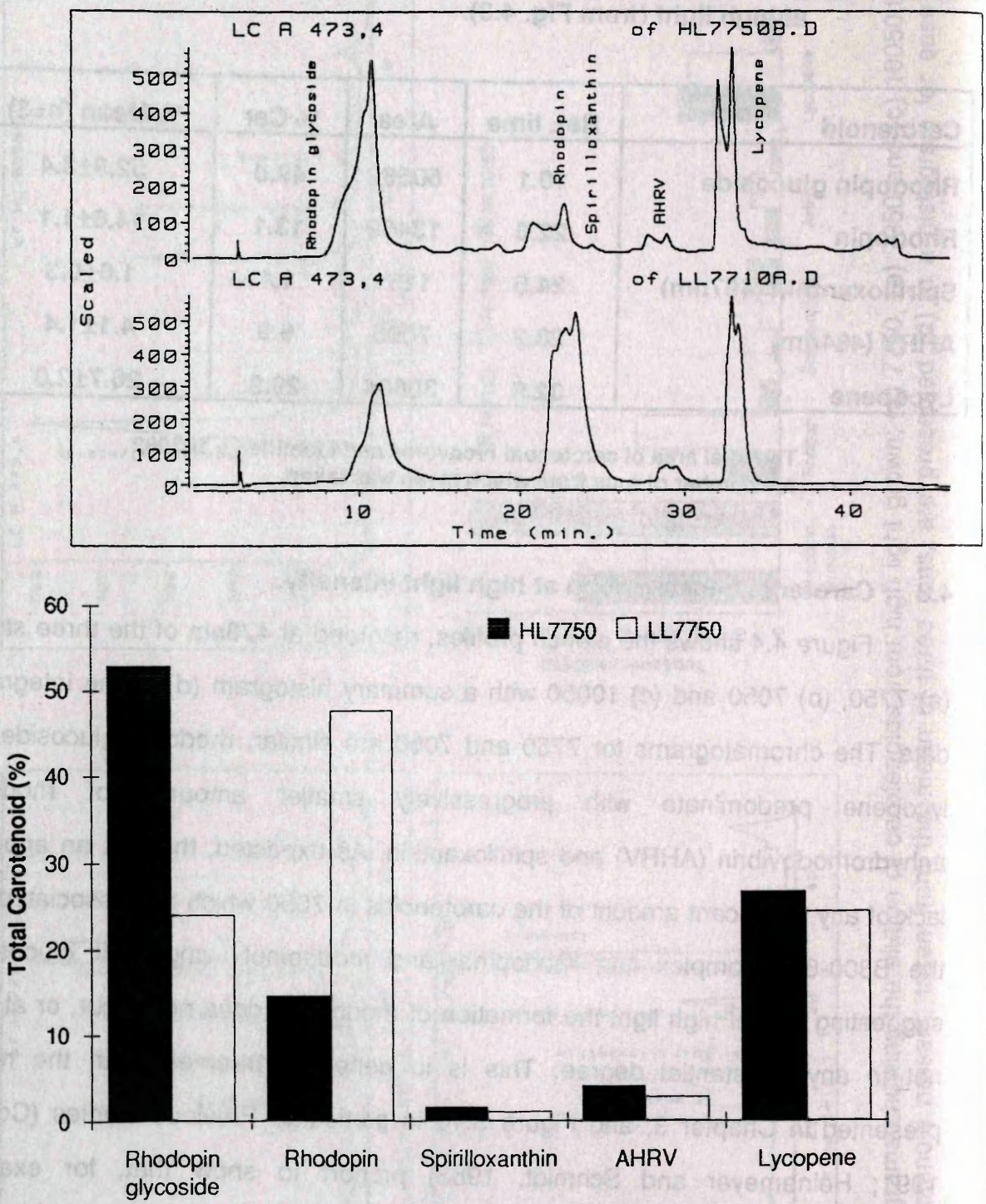



**Figure 4.5:** Comparison of the total carotenoid composition of *Rps. acidophila* 7750 grown at high (160µmols/s/m²) and low (10µmols/s/m²) light intensities. The change in the ratio of rhodopin:rhodopin glycoside is evident in the differences between the chromatograms (top) or graphically in the histogram (bottom). The data at low light was compiled from two repeated chromatograms, as opposed to the usual three.

enough to suppress this complex and rhodopinal formation. Thus this anomaly can be assigned to differences between laboratories in the definition of 'high light'.

The chromatogram for 10050 shows this strain is markedly different to the other two, with rhodopin glucoside accounting for almost eighty percent of the total carotenoid, with the other four present in roughly equal amounts. Some difference between 10050 and the other two strains could have been predicted from the slight orange colour difference when the cells are growing in liquid culture; as yet though, the physiological reason for the virtual predominance of rhodopin glucoside in this strain is unknown.

## 4.4 Effect of low light on the carotenoid composition of *Rps acidophila* strain 7750.

*Rps. acidophila* 7750 was cultured at 10µmols/s/m² to induce the expression of the B800-820 complex in place of B800-850. Carotenoids were then extracted to establish what change, if any, had occurred. The data from the chromatograms is presented in Figure 4.5.

Upon the shift to low light there is no significant change in the amount of lycopene, AHRV or spirilloxanthin, however, there is an exchange in the rhodopin content. At high light the amount of rhodopin and rhodopin glucoside is 14.6 and 52.9% respectively whereas the low light adapted cells contain 47.6 and 24.1%. This exchange is possibly due to a reduction in the rate of gluconeogenesis which prevents the production of metabolically expensive glucose moieties, at a time when the cell is under stress due to the reduction in the available light.

## 4.5 Effect of low light on the carotenoid composition of *Rps acidophila* strain 7050.

The effect of progressively decreasing the light intensity on both the carotenoid composition and the peripheral antenna of *Rps. acidophila* 7050 has been described in Chapter 3.3. Carotenoids were extracted from the

chromatophores that produced each spectra in Figure 3.9 and chromatograms from high and low light (10μmols/s/m²) are given in Figure 4.6. The appearance of the purple carotenoid rhodopinal and particularly rhodopinal glucoside can easily be seen along with concomitant decrease in the amount of rhodopin, its glucoside, AHRV, spirilloxanthin and lycopene. The main carotenoid content at each light intensity has been plotted in Figure 4.7. From this graph three phases can be distinguished: both the growth room culture (HL) and that grown at 160μmols/s/m², have a carotenoid composition typical of high light cells. The spectra for these chromatophores (Fig. 3.9) indicate the peripheral antenna is exclusively B800-850. The cells grown at 20, 10 and 4μmols/s/m² have completed their adaptation to the reduced light intensity with the result that across this range very little alteration in their antenna complement occurs; at these lowered light intensities the peripheral antenna has switched to exclusively B800-820. It is between these two regions that the adaptation to low light takes place; examination of the data points at 110 and 40μmols/s/m² reveals the sample contains both rhodopin and rhodopinal (and their respective glucosides). In the case of the 40μmols/s/m² preparation the amounts of these carotenoids are neither equivalent to those in high or low light cells respectively. Moreover, the spectra equivalent to this light intensity in Fig. 3.9 indicates the peripheral antenna is a mixture of both B800-850 which is primarily associated with the rhodopin, and B800-820, primarily associated with rhodopinal. Although it should be mentioned that to the eye the culture at this light intensity appeared equally as purple as those at lower light. The 110μmols/s/m² culture also contains a small amount of rhodopinal glucoside. The absorption spectra suggests that there is no B800-820 insertion as yet into the membrane, however, the carotenoid region of the spectrum has lost a great deal of its fine structure. Thus these results confirm the hypothesis outlined in the previous chapter that the control of the carotenoid 'switch' i.e., rhodopin to rhodopinal, and the antenna 'switch' i.e., B800-850 to B800-820 are not synchronous, rather the change in carotenoid precedes that of the peripheral antenna as the available light decreases.

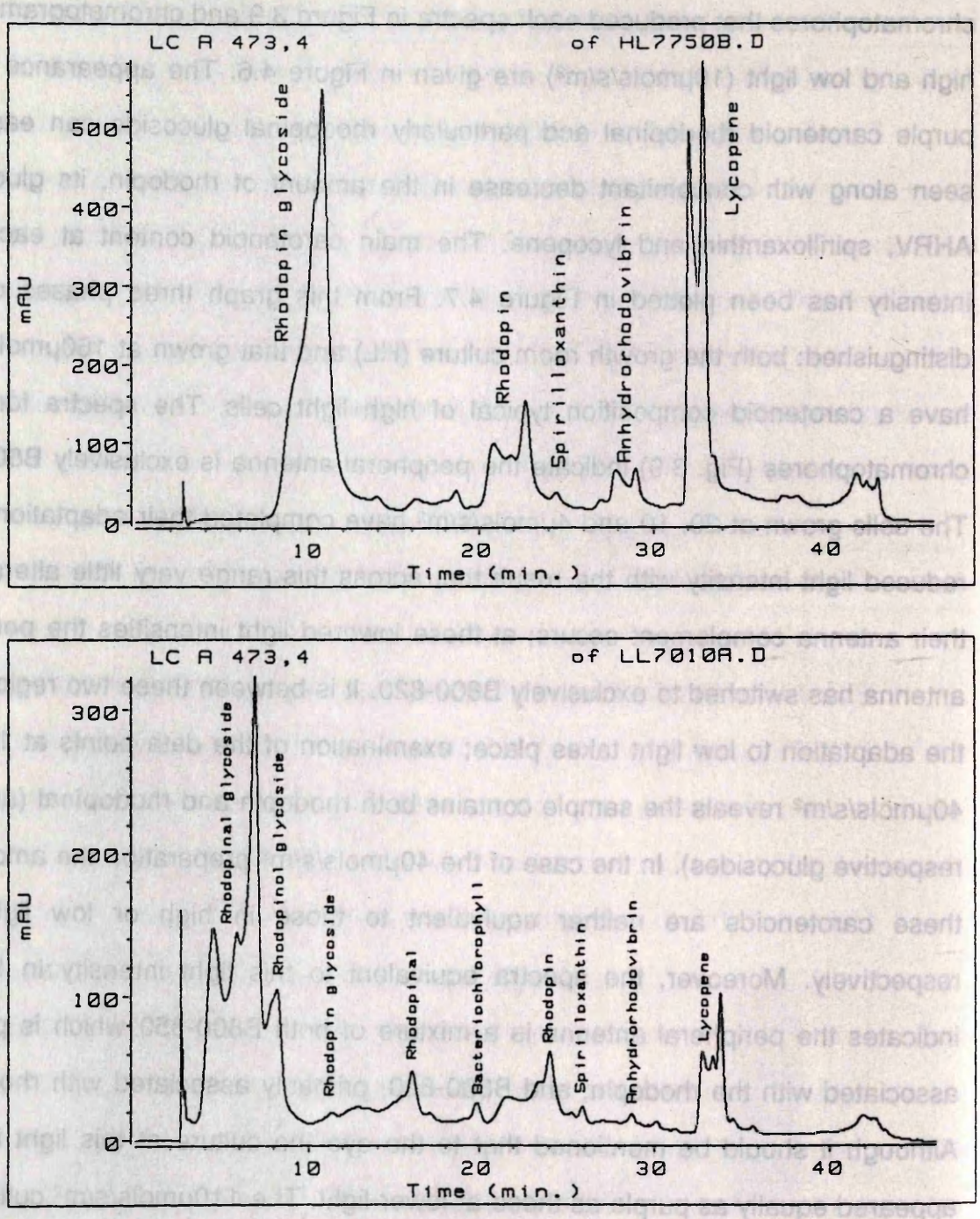



**Figure 4.6:** Chromatograms showing the effect of decreasing the light intensity on the carotenoid composition for *Rps. acidophila* 7050. The high light culture (top) contains the orange carotenoids rhodopin and its glycoside whereas the low light culture (bottom) contains the purple carotenoids rhodopinal and rhodopinal glycoside.

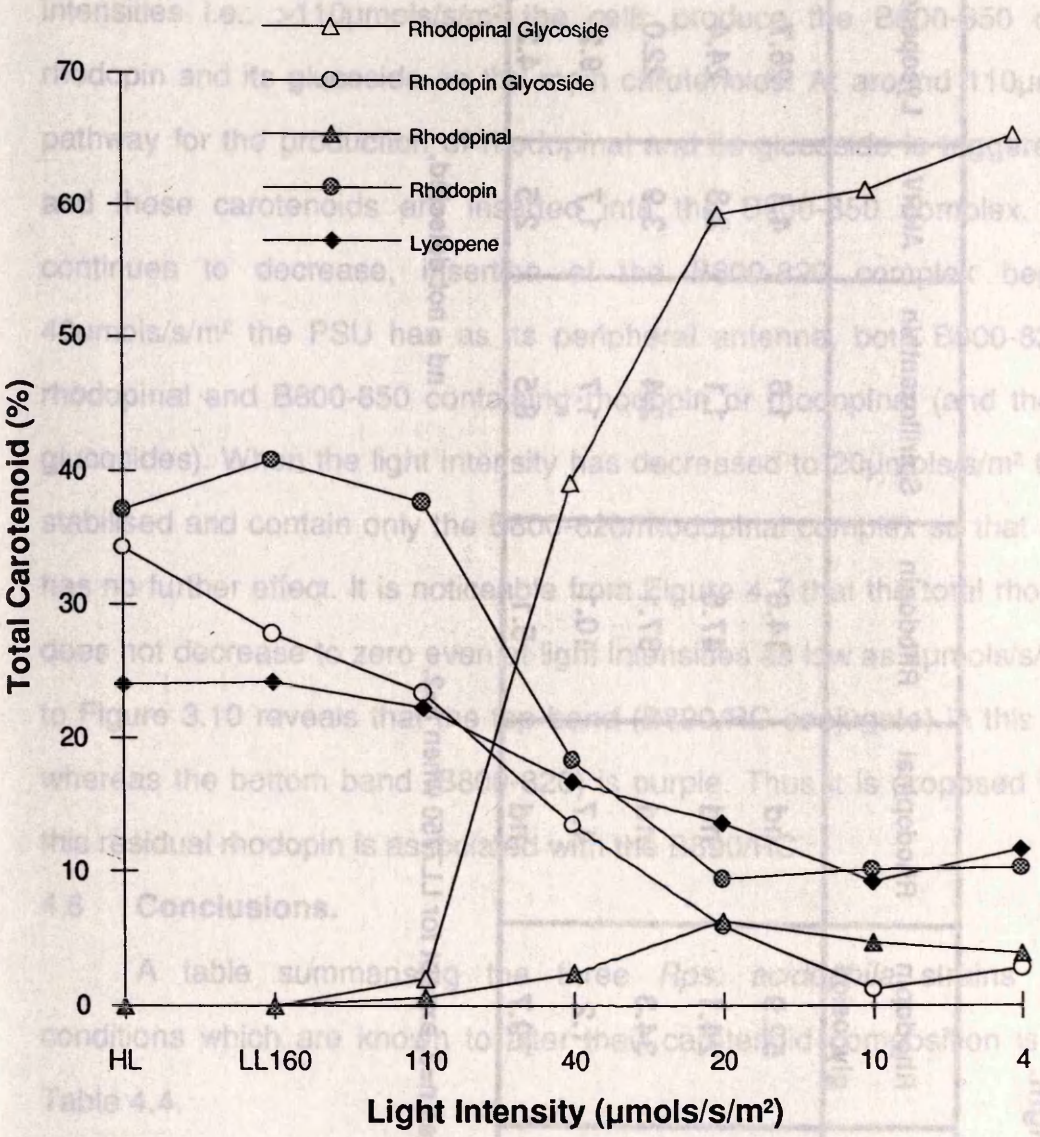



**Figure 4.7:** Effect of light intensity on carotenoid production for *Rps. acidophila* 7050. The data points are given as a percentage of the total carotenoid; HL, LL160 and 10 are the mean attained from three chromatograms, the remaining data points are from one run only.

**Table 4.4:** **Percentage carotenoid composition for *Rps. acidophila* strains under high (160µmols/s/m²) and low (10µmols/s/m²) light.**

| | Rhodopinal glycoside | Rhodopinol glycoside | Rhodopin glycoside | Rhodopinal | Rhodopin | Spirilloxanthin | AHRV | Lycopene |
|---|---|---|---|---|---|---|---|---|
| HL7750 | nd | nd | 52.9 | nd | 14.6 | 1.6 | 4.1 | 26.7 |
| LL7750 | nd | nd | 24.1 | nd | 47.6 | 1.1 | 2.8 | 24.4 |
| HL7050 | nd | nd | 34.3 | nd | 37.1 | 2.4 | 3.9 | 22.0 |
| LL7050 | 61.2 | 6.7 | 1.2 | 4.7 | 10.1 | 1.7 | 1.7 | 9.2 |
| HL10050 | nd | nd | 79.7 | nd | 5.1 | 8.5 | 2.5 | 4.2 |

In all cases n=3, except for LL7750 when n=2. nd = not detected.

To summarise briefly the effect of light intensity on 7050: at high light intensities i.e., >110µmols/s/m² the cells produce the B800-850 complex with rhodopin and its glucoside as the main carotenoids. At around 110µmols/s/m² the pathway for the production of rhodopinal and its glucoside is triggered (Fig. 1.6b) and these carotenoids are inserted into the B800-850 complex. As the light continues to decrease, insertion of the B800-820 complex begins until at 40µmols/s/m² the PSU has as its peripheral antenna, both B800-820 containing rhodopinal and B800-850 containing rhodopin or rhodopinal (and their respective glucosides). When the light intensity has decreased to 20µmols/s/m² the cells have stabilised and contain only the B800-820/rhodopinal complex so that any decrease has no further effect. It is noticeable from Figure 4.7 that the total rhodopin content does not decrease to zero even at light intensities as low as 4µmols/s/m², reference to Figure 3.10 reveals that the top band (B890/RC conjugate) in this well is brown whereas the bottom band (B800-820) is purple. Thus it is proposed that (most of) this residual rhodopin is associated with the B890/RC.

## 4.6 **Conclusions.**

A table summarising the three *Rps. acidophila* strains grown under conditions which are known to alter their carotenoid composition is presented in Table 4.4.

Strain 7050 has the most dramatic effect, in that under low light a completely new carotenoid, rhodopinal and its glucoside are synthesised. This carotenoid has a higher efficiency for energy transfer when acting as an accessory light harvesting pigment than its precursor, rhodopin, and this in concert with insertion of B800-820 into the membrane enables this strain to grow well under low light.

Strain 7750 at low light also synthesises this complex and redistributes the proportions of rhodopin and its glucoside without synthesising novel ones.

Strain 10050 does not make any adjustment to its carotenoid composition (from 3.5), under low light, although it is conceivable that it could redistribute the

proportions of rhodopin and its glucoside. Its carotenoid composition at high light consists of approximately 80% rhodopin glucoside.

# Chapter Five

## Effect of peripheral antenna type on exciton migration within the photosynthetic unit.

## 5.1 Introduction.

In general, the study of emitted fluorescence is a valuable tool when examining the properties of photosynthetic membranes. It was used in this chapter, in particular, to try and ascertain the exciton migration patterns with regard to the antenna organisation of *Rps. acidophila.* The two previous Results chapters were based on the assumption that insertion of the B800-820 complex into the ICM was advantageous to the bacterium when growing under non-favourable conditions, and in so doing the light-harvesting capability of the cell would be enhanced. Evidence will now be produced to support this assumption.

It has been recognised for many years that fluorescence yield depends on the intensity of the exciting light. This can be explained by the assumption that, at least in the Rhodospirillaceae, reaction centres (RC) where the primary donor is reduced are more efficient energy traps than RCs where the primary donor is oxidised. An exciton has in a given time a certain probability that it will decay through fluorescence, therefore, if an exciton arrives at an oxidised RC it is able to continue its random walk until it finds a reduced RC, or if the exciting light is too high and all RCs to which the exciton migrates are oxidised, the probability of decay through fluorescence is enhanced and the result will be an increased fluorescence yield. The two states of the RC, reduced and oxidised, are called 'open' and 'closed' respectively.

All experiments in this chapter were performed in the Dept. of Biophysics, Huygens Laboratory, University of Leiden in collaboration with Dr. G. Deinum, under the supervision of Prof. J. Amesz.

## 5.2 Fluorescence spectra obtained at low excitation intensity.

*Rps. acidophila* was cultured under three different growth regimes that have been proven in Chapter 3. to produce cultures containing different peripheral antenna complexes. Absorption spectra of the three different cultures used in the experiments are shown in Figure 5.1, they are (a) chromatophores from strain 7750

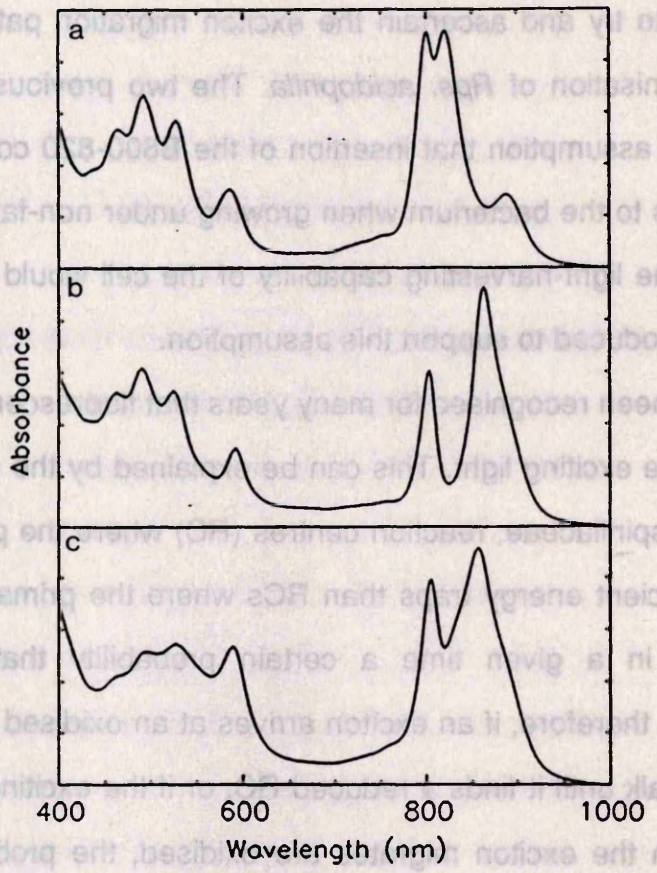



**Figure 5.1**: Absorption spectra of *Rps. acidophila* chromatophores used in the experiments detailed in this chapter:

**(a)** Strain 7750 grown at 50µmols/s/m², 22°C, abbreviated to LT7750. These chromatophores contain solely B800-820 as peripheral antenna
**(b)** Strain 7750 grown at 160µmols/s/m², 30°C, abbreviated to HL7750. These chromatophores contain solely B800-850 as peripheral antenna
**(c)** Strain 7050 grown at 50µmols/s/m², 30°C, abbreviated to LL7050. These chromatophores contain both B800-850and B800-820 as peripheral antenna

cultured at 22°C with low light [abbreviated to LT7750], therefore, the peripheral complex consists only of B800-820, (b) chromatophores from 7750 cultured at 30°C with high light, i.e., normal growth room conditions [abbreviated to HL7750], therefore, the peripheral complex contains solely B800-850, finally, (c) chromatophores from 7050 grown at a light intensity, [approx. 50µmols/s/m², abbreviated to LL7050] that produces a mixed peripheral antenna complement of both B800-850 and B800-820.

The fluorescence emission spectra for *Rps. acidophila* 7750 grown with low light at 22°C, LT7750, are shown in Figure 5.2a. The fluorescence spectra were recorded in the presence of continuous background illumination (solid line) or in the absence of background illumination (dotted line) and are characterised by the presence of two peaks with maxima near 840nm and 903nm, which can be ascribed to fluorescence originating from B800-820 and B890 respectively. The intensity of the peak at 840nm was independent of the redox state of the reaction centres, in contrast to the peak at 903 which showed an increase in fluorescence by a factor of about 2.1 upon oxidation of P-870. These observations suggest that excitons harvested by the B800-820 complex and passed on to the B890 complex by the prevailing energy gradient are unable to complete the reverse process, otherwise the increase in fluorescence would have been seen also at 840nm. Thus, this has the effect of directly funnelling excitons into B890 complex (and on to the RC), the large energy gap between B800-820 and B890 (0.11eV) effectively acts as a barrier to the backward flow of excitons. This result agrees with the hypothesis that only B890 is directly connected to the reaction centre and that reversed i.e., 'up-hill' energy transfer from B890 to B800-820 does not occur.

The same measurements were performed on the HL7750 sample and the fluorescence spectra with P-870 oxidised (solid line) or reduced (dotted line) are shown in Figure 5.2b. The spectra show a peak at about 899nm and a weak shoulder at approx. 870nm originating from B890 and B800-850 respectively. Contrary to the previous figure there was an increase in fluorescence intensity over

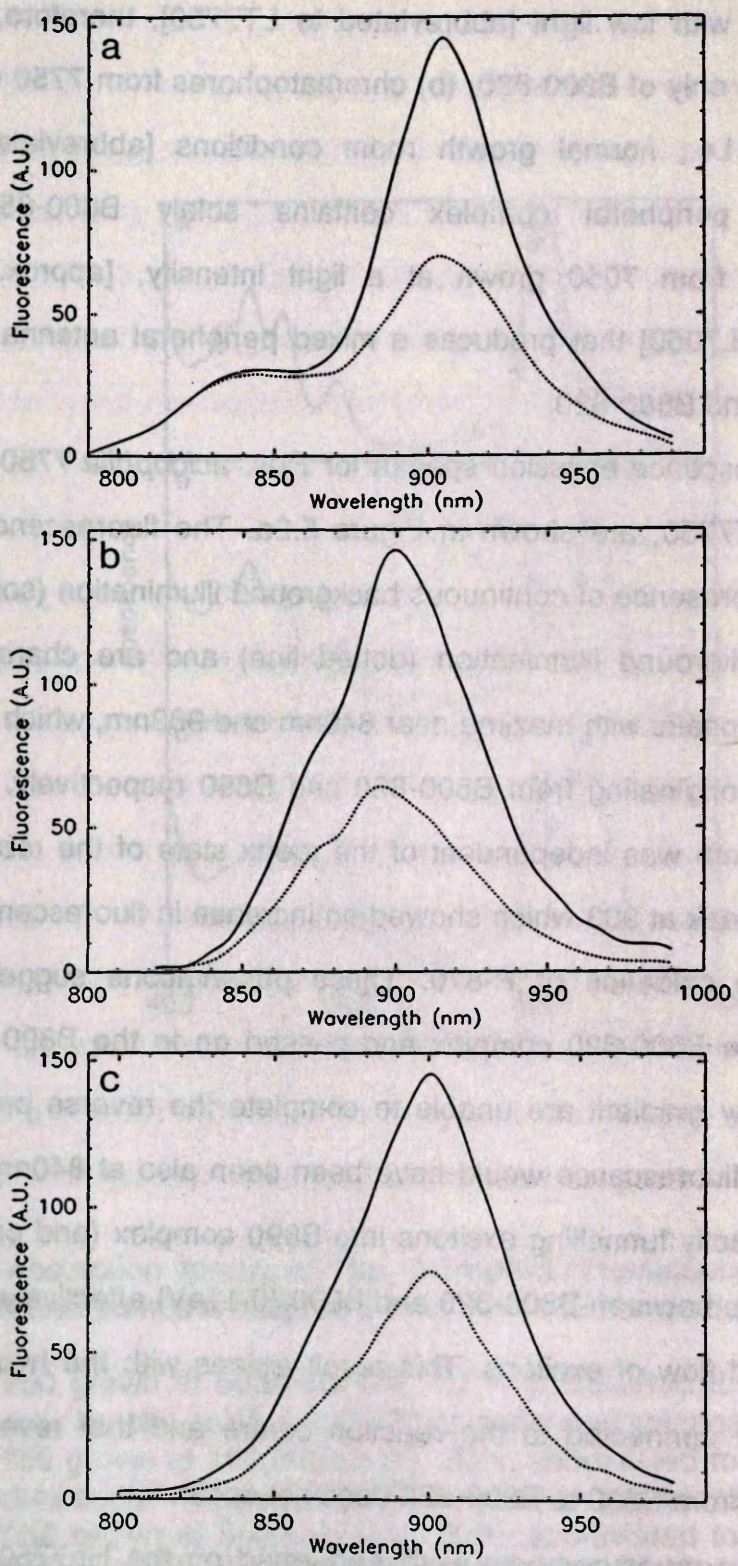



**Figure 5.2:** Emission fluorescence spectra of *Rps. acidophila* chromatophores. The spectra were obtained with a low intensity xenon flash with P-870 reduced (dotted line) and with P-870 oxidised (solid line) by means of continuous background illumination. A U., arbitrary units. **(a)** LT7750. **(b)** HL7750. **(c)** LL7050.

the whole spectrum upon oxidation of P-870, by a factor which varied from 2.0 to 2.4 between 870 and 970nm. This suggests that the fluorescence of both B800-850 and B890 is affected by the redox state of the RC, indicating that excitons can be transferred 'up-hill' from B890 to B800-850 and that B800-850 is not an efficient barrier to the backward flow of excitons. In other words, this complex, unlike B800-820, is not able to funnel in a unidirectional manner excitons into the RC. The difference in the fluorescence increase is due to the extent of overlap between the fluorescence and absorption integrals from the two complexes.

Figure 5.2c shows the fluorescence spectrum of LL7050 chromatophores which contain all three complexes, B800-820, B800-850 and B890. There is a maximum of fluorescence at 900nm associated with B890. A shoulder near 870nm, which was less pronounced than in Figure 5.2b, may be ascribed to B800-850. The increase of fluorescence by about a factor of 2.0 was independent of the wavelength. Apart from the difficulty in clearly distinguishing B800-850 fluorescence from that of B890 the fluorescence properties were very similar. Any fluorescence from the B800-820 complex has probably been masked due to two reasons; the proportion of B800-820 to B800-850, judging by the absorption spectrum, is rather low and the red absorbing Bchl in this complex has its maxima closer to 830nm than 820nm, therefore, its fluorescence will tend to merge more with that of the B800-850 complex compared with the B800-820 complex from 7750.

## 5.3 **Fluorescence spectra obtained at high excitation intensity.**

Fluorescence spectra of these samples were also obtained using a high intensity laser flash for excitation and compared with those previously obtained using xenon flash excitation. The results of the measurements on LT7750 for the xenon (dotted line) and the laser flash (solid line) are given in Figure 5.3a. Both spectra were obtained under conditions when P-870 was oxidised. The emission around 840nm was much more pronounced in the laser flash spectrum than in that of the xenon flash. The difference between the two spectra reflects the different

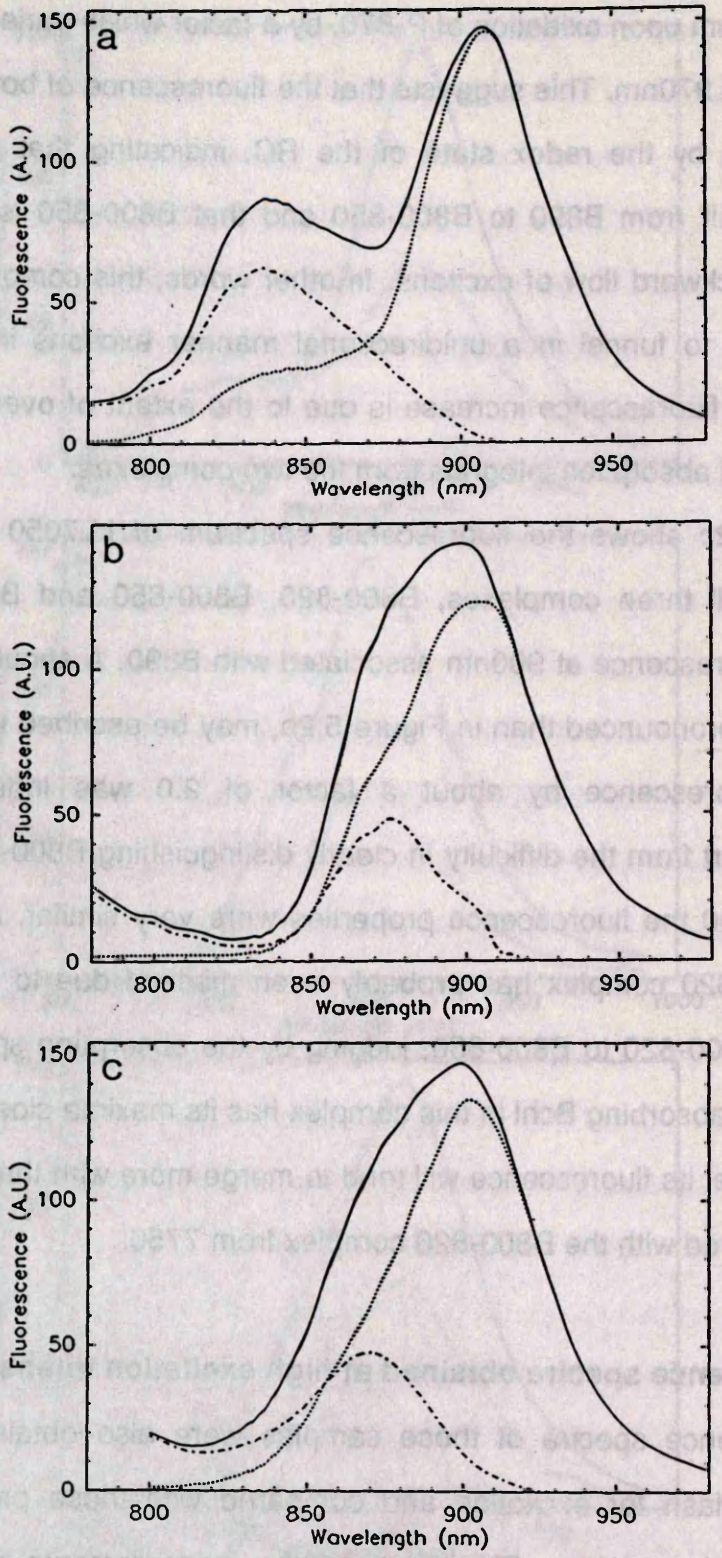



**Figure 5.3:** Relative fluorescence yield as a function of wavelength for the *Rps. acidophila* chromatophores, with xenon flash excitation (dotted line) and with an intense laser flash of 1mJ/cm² (solid line). The latter spectrum was multiplied by about 40 in order to normalise the spectra at wavelengths longer than 910nm. The difference of the two spectra is given by the broken line. **(a)** LT7750. **(b)** HL7750. **(c)** LL7050.

extent of singlet-singlet annihilation (see 1.7.4) in the two pigment complexes, resulting from unequal exciton densities due to rapid energy transfer from B800-820 to B890. Subtracting the two spectra after normalisation in the region above 910nm, where only B890 fluorescence occurs, yields a band with a maximum at 835nm which can be ascribed to B800-820. This is caused by the fact that energy transfer occurs from B800-820 to B890 which enhances the extent of singlet-singlet annihilation taking place in the B890 complex.

Similar measurements on HL7750 are shown in Figure 5.3b. As with the previous figure the quenching was strongest at wavelengths above 910nm. Subtraction now yielded a band at 875nm, which may be attributed to B800-850, again caused by a higher extent of singlet-singlet annihilation in the B890 complex due to energy transfer from the B800-850 to the B890 complex.

Essentially the same results as those with HL7750 were obtained with a 7050 culture that contained both types of peripheral complex, are shown in Figure 5.3c. However, the laser flash spectrum extended somewhat further towards the blue than that of HL7750, indicating that in these chromatophores some of the fluorescence originates from the B800-820 complex.

## 5.4 **Annihilation measurements at specific wavelengths.**

All calculations in this section were performed by Dr. G. Deinum, University of Leiden. The relative fluorescence yield as a function of the laser flash energy for culture LT7750, containing B890 and B800-820 only is shown in Figure 5.4a. The measurements were performed at 840nm and 930nm, respectively, in the presence of continuous background illumination to keep P-870 oxidised. The measurements at 930nm may be used to obtain information about the domain size of B890 by applying equation (20) of Paillotin *et al.,* (1979). Generating curves according to that equation for different values of the parameter r and fitting curves to the data, established that the best fit at 930nm was obtained with r = 0.7. Such a small value for r means that the relative efficiency of annihilation per pair of excitons within one

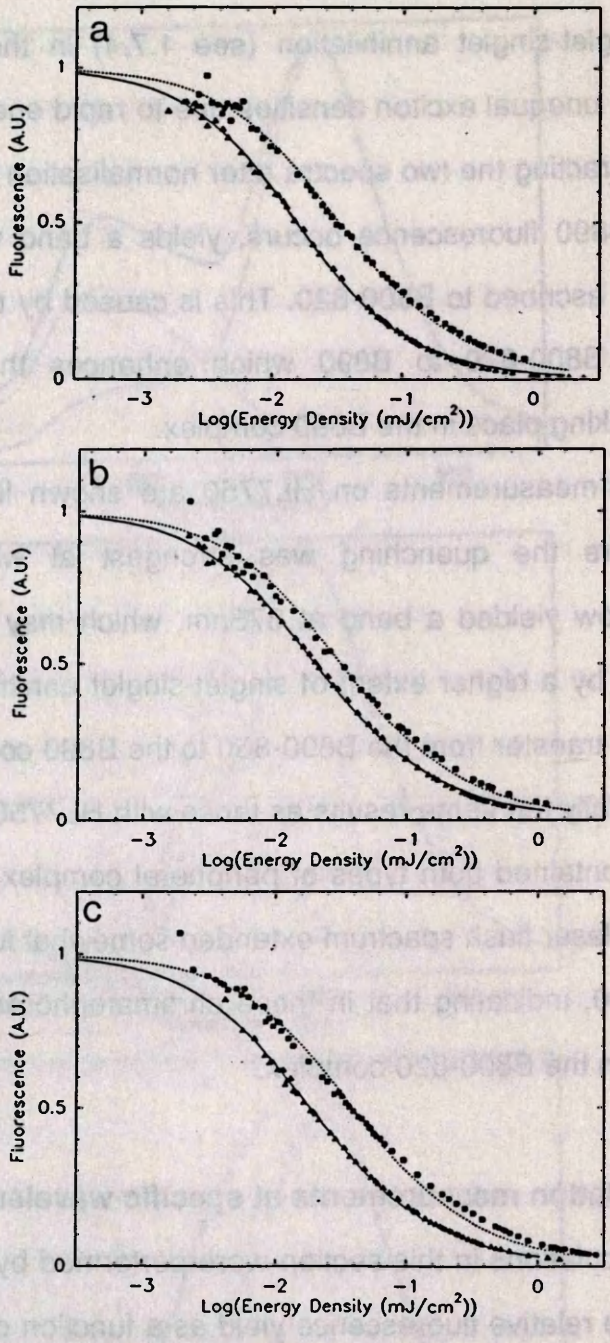



**Figure 5.4:** The relative yield of fluorescence plotted as a function of the incident energy density of the excitation flash for *Rps. acidophila* chromatophores. The curves are normalised at low energy densities.
**(a)** LT7750 ■, detected at 930nm and fitted with Eqn. 20 from Paillotin *et al.*,(1979) with r = 0.7; ▲, detected at 840nm. The dotted curve gives the best fit for 840nm when r = 2.
**(b)** HL7750 ■, detected at 930nm and fitted with Eqn. 20 from Paillotin *et al.*,(1979) with r = 2; ▲, detected at 860nm. The dotted curve gives the best fit for 860nm when r = 2.
**(c)** LL7050 ■, detected at 930nm and fitted with Eqn. 20 from Paillotin *et al.*,(1979) with r = 2; ▲, detected at 860nm. The dotted curve gives the best fit for 860nm when r = 2.

domain is high, this implies a small domain size and corresponds to a domain of 80 ± 20 Bchls in B890. However, this has neglected to take into account annihilation occurring in B800-820, therefore, the true domain size must be smaller. The variables that were defined in order to calculate a corrected value for the domain size are outlined in Deinum *et al.*, (1991): by this method a r-value of 0.3 ± 0.2 was calculated which corresponded to a domain size 50 ± 20 Bchls for B890. In spite of the uncertainty involved in this calculation, the result indicates that this antenna forms small units, the size of which is comparable to the number of Bchls per RC determined biochemically by Dawkins *et al.*, (1988).

Contrary to the measurements at 930nm, those at 840nm could not be simulated by any value of r, because the slope of the experimental curve was smaller than of any theoretical curve. Figure 5.4a (dotted line) shows a 'best fit' with r = 2; curves for higher values are very similar. Deinum, (1991) has shown that this effect may occur when the lifetime of the excitons is comparable to the length of the laser pulse. A short lifetime of the excitons in B800-820 originates from rapid energy transfer from this complex to B890.

Results of measurements on the HL7750, with B890 and B800-850 only, are shown in Figure 5.4b. The measurements were performed at 930 and 860nm respectively, with closed RC. For the fluorescence recorded at 860nm no proper fit could be obtained due to the small slope of the curve, presumably for the same reason as given for culture LT7750. Moreover the fluorescence recorded at this wavelength contains an unknown amount of fluorescence emitted by B890, as is clear from Fig. 5.3b. The measurements performed at 930nm could be fitted $r \geq 2$, although at this wavelength most annihilation will take place in B880, the effects caused by energy transfer from B890 to B800-850 cannot be ignored for this culture, and as such cannot be analysed properly by the equation of Paillotin *et al.*, (1979), which is only valid for spectrally homogeneous systems. If, however, the inhomogeneity is disregarded it can be calculated that the number of connected Bchls in B890 is at least 200, which is comparable to the domain sizes previously

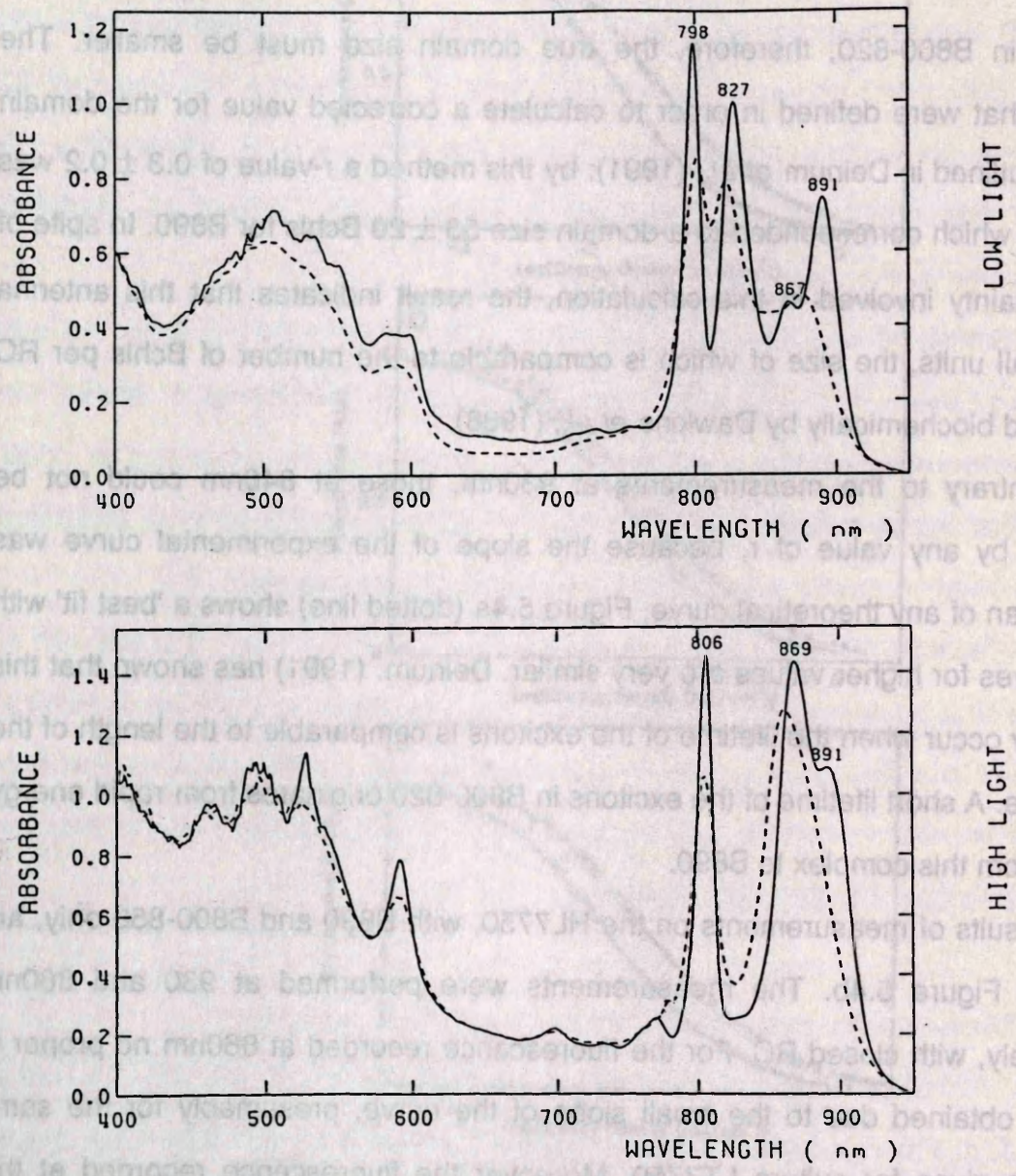



**Figure 5.5:** Absorption spectra of *Rps. cryptolactis* chromatophores; solid line, 70K; dashed line, 300K. **(a)** The low light preparation is analogous to *Rps. acidophila* 7750 when grown at 22°C i.e., LT7750. (This is not strictly true as these chromatophores contain a small amount of B800-850). **(b)** The high light preparation is analogous to *Rps. acidophila* 7750 when grown at 30°C under normal growth room conditions i.e., HL7750. The difference in the carotenoid composition of the cells at these two growth conditions is (more) apparent from the 70K spectra.

determined for *Rb. sphaeroides,* Vos *et al.,* (1986) and *Rb. capsulatus,* Bakker *et al.,* (1983).

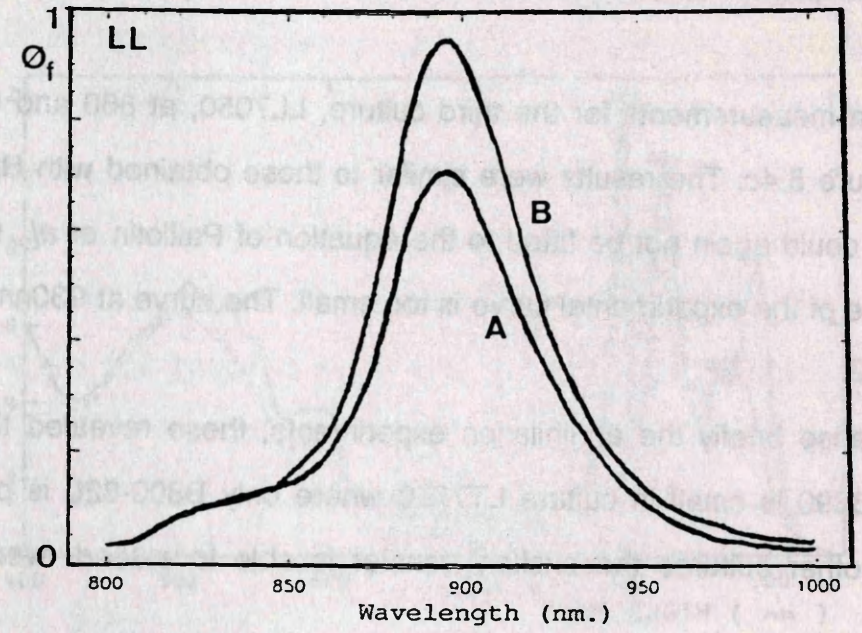
Annihilation measurements for the third culture, LL7050, at 860 and 930nm are shown in Figure 5.4c. The results were similar to those obtained with HL7750. Those at 860nm could again not be fitted to the equation of Paillotin *et al.,* (1979), because the slope of the experimental curve is too small. The curve at 930nm could be fitted with $r \geq 2$.

To summarise briefly the annihilation experiments, these revealed that the domain size of B890 is small in culture LT7750 where only B800-820 is present, whereas in the other cultures the exciton transfer is able to extend over larger distances.

## 5.5 Emission fluorescence spectra obtained from *Rps. cryptolactis.*

*Rps. cryptolactis* is a recently isolated thermotolerant member of the Rhodospirillaceae which closely resembles *Rps. acidophila* 7050 with regard to the changes in its peripheral antenna in response to growth at reduced light intensity i.e., production if a B800-820 complex in place of B800-850 alongside a change in the carotenoid composition.

The absorption spectra for this species when grown at low and high light are given in Figures 5.5a and 5.5b respectively. It is evident that at low light the peripheral antenna consists mainly of B800-820 although a small amount of B800-850 is also present. At the time that these experiments were performed the growth conditions necessary to culture this species at low light so that they were totally devoid of B800-850 had not been perfected. At high light the cells contain only B800-850. Thus, it is apparent that species provides an ideal opportunity to validate the conclusions reached with *Rps. acidophila* regarding exciton migration in a spectrally inhomogeneous antennae system. Using the same experiments that were described for *Rps. acidophila,* the properties of *Rps. cryptolactis* high light (HL), B800-850 and low light (LL) B800-820 containing chromatophores were measured



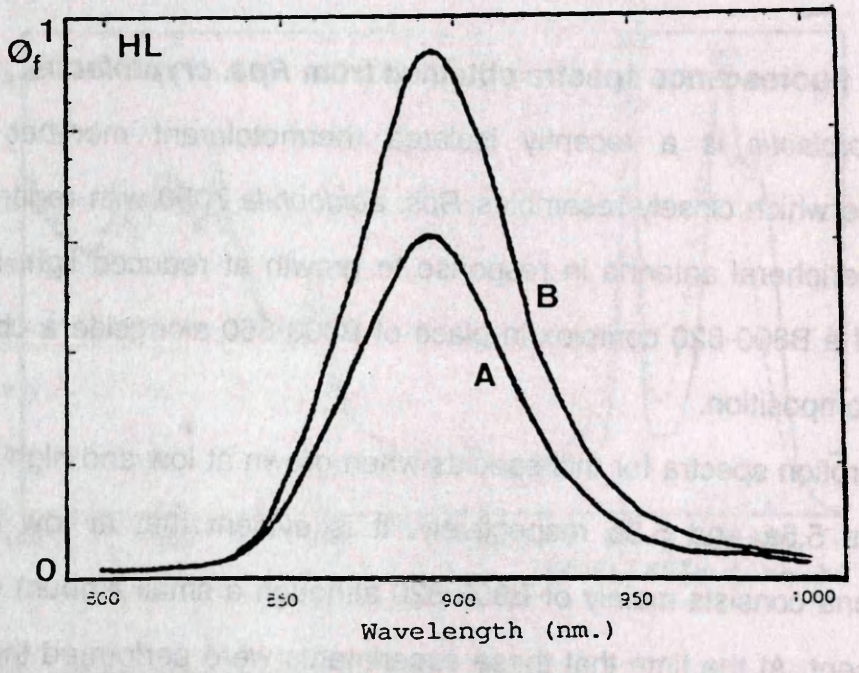




**Figure 5.6:** Fluorescence emission spectra of *Rps. cryptolactis* chromatophores; **(LL)** The low light spectra are very similar to those obtained in Fig. 5.2a for *Rps. acidophila,* they have been excited with a low intensity xenon flash, **(A)** with the RCs open (reduced state), and **(B)** with the RCs closed (oxidised state). This nomenclature is retained for the high light **(HL)** chromatophores, which were similar to those obtained in Fig. 5.2b for *Rps. acidophila.*

by emission fluorescence. The resultant spectra, presented in Figure 5.6, illustrate that the low light spectra with open traps contain a shoulder at approximately 830nm corresponding to the B800-820 complex, in addition to the main peak at 896nm. Upon oxidation of the primary donor there was an increase in the fluorescence yield of about 1.4 at 896nm originating from B890, whereas the fluorescence yield originating from the B800-820 complex did not alter. This result is similar to that previously presented in Fig. 5.2a. and indicates an analogous situation in happening in this species as for *Rps. acidophila* where exciton transfer from B800-820 to B890 i.e., 'up-hill' cannot occur.

The closed traps emission fluorescence spectrum for the high light sample shows an increase across the whole range of wavelengths against the open spectrum, Figure 5.6b, and indicates that energy transfer from B800-850 to B890 can occur. This Figure for *Rps. cryptolactis* is similar to that already presented for *Rps. acidophila* in Fig. 5.2b and provides further evidence that the exciton migration patterns are the same in both species. In other words, the concerted funnelling of excitons by the B800-820 complex into the B890 complex and on to the RC (and the lack of funnelling by B800-850) is result of intrinsic properties conferred by the different types of complex and is not due to some inherent property of any individual species.

Annihilation experiments which could provide information regarding the sizes of the domains are presently being performed as part of an ongoing research project in collaboration with the Dept. of Biophysics, Huygens Laboratory, University of Leiden.

## 5.6 Conclusions.

These results show that the patterns of energy migration in *Rps. acidophila* and *Rps. cryptolactis* are strongly affected by the types of peripheral antenna complexes present in the membrane. From the fluorescence measurements as a function of the redox state of P-870 the conclusion is apparent that excitons once arrived in B890, can be transferred back to B800-850, but not to B800-820, and that

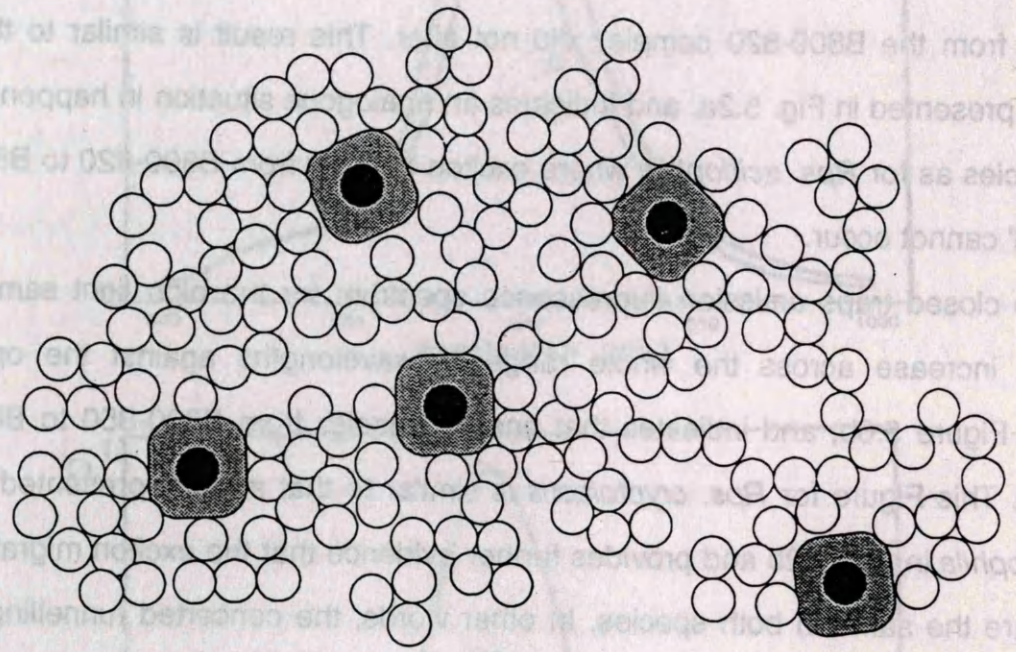


**Figure 5.7:** Model of the photosynthetic antenna of *Rps. acidophila* and *Rps. cryptolactis.* The peripheral antenna (B800-820 and/or B800-850, depending on the culture conditions) are indicated by the open circles, the core antenna (B890) by the grey rounded boxes and the reaction centres by the black circles. Compare with the Monger and Parson, (1977) [see Fig. 1.10] model for *Rb. sphaeroides.*

only the B890 complex is connected to the RC. This latter effect is likely to be caused by the large energy gap between B890 and B800-820 (0.11eV) and can be explained by assuming a small overlap integral of the B890 fluorescence band and the absorption band of the B800-820 complex in the Förster equation. The reverse process, energy transfer from either B800-850 or B800-820 to B890 turned out to be very efficient.

Singlet-singlet annihilation measurements showed that in cells containing only B890 and B800-820 i.e., *Rps. acidophila* 7750 grown with low light at 22°C, that the domain size of B890 is small, approx. 50±20 Bchl. This is consistent with the emission fluorescence spectra obtained previously and also with the number of Bchls per reaction centre determined biochemically by Dawkins, (1988). In high light *Rps. acidophila* 7750 cells containing the B800-850 complex, energy transfer can take place over at least 200 Bchl molecules, again consistent with the emission fluorescence spectra for this growth regime, in that excitons are not confined to the B890 complex. (The equivalent estimation for *Rps. acidophila* 7050 was not possible because of the heterogeneity present in the antenna). The model presented in Figure 5.7 is able to account for all the results presented in this chapter. In this model the B890/RC conjugate are separated by either B800-850 or B800-820 and it differs from that proposed by Monger and Parson, (1977) [see Fig. 1.10] for *Rb. sphaeroides* which consists of 'channels' of B875 connecting the reaction centres. These results show that, at least for *Rps. acidophila* 7750 containing only B800-820, this latter model does not apply. The model presented in Figure 5.7 also explains the results obtained with the other samples since B800-850 is not an efficient barrier and so fluorescence will depend on the redox state of P-870 and larger domain sizes will be observed.

When these results are viewed in connection with those obtained in the last two chapters the physiological significance of B800-820 expression is apparent. The consequence of B800-820 as the peripheral complex is that excitons harvested by this complex are funnelled directly to B890, hence light harvesting is maximised,

# Chapter Six

## Development of a genetic system for the introduction of cloned DNA into the Rhodospirillaceae.

## 6.1 Introduction.

This chapter was intended to evaluate the feasibility of utilising conjugal transfer to introduce cloned DNA from *E. coli* into *Rps. acidophila* as a first step in the molecular investigation of peripheral antenna genes and their regulation. This assessment was extended to two other species which also have interesting peripheral antenna complements: *Rps. palustris,* which produces a different B800-850 complex at low light (see 1.4) and *Rhodospirillum rubrum,* which has no peripheral antenna. Genes conferring antibiotic resistance were used as phenotypic markers: as a prerequisite, therefore, to any mating experiments it was necessary to determine the intrinsic susceptibility of each species to any particular antibiotic. This would then enable the ability of each mobilising plasmid i.e., the cloning vector, to be transferred from the donor to the recipient to be gauged via their respective transfer efficiencies. Furthermore, as transposon Tn5 mutagenesis is an invaluable tool for identifying and mapping genes, the feasibility of introducing Tn5 on a 'suicide' plasmid to each species using this system was also evaluated.

Because these techniques are well established for *Rb. sphaeroides* this species will be used as a benchmark in this chapter, against which the success or otherwise of the mating experiments will be assessed. The relevant characteristics of each plasmid and the genotypes of all *E. coli* strains used in this chapter are listed in Appendix D.

## 6.2 Susceptibility of Rhodospirillaceae to antibiotics.

Figure 6.1a and 6.1b show the effect of tetracycline on the four Rhodospirillaceae species under study. The equivalent graphs for the other antibiotics are presented in Appendix. C. These results can be summarised as follows:

***Rb. sphaeroides***: This species proved susceptible, i.e., 100% killing, to kanamycin (Km), neomycin (Nm) and streptomycin (Sm) at concentrations <1µg/ml. However 10µg/ml was required for tetracycline (Tc). Previous reports have suggested that

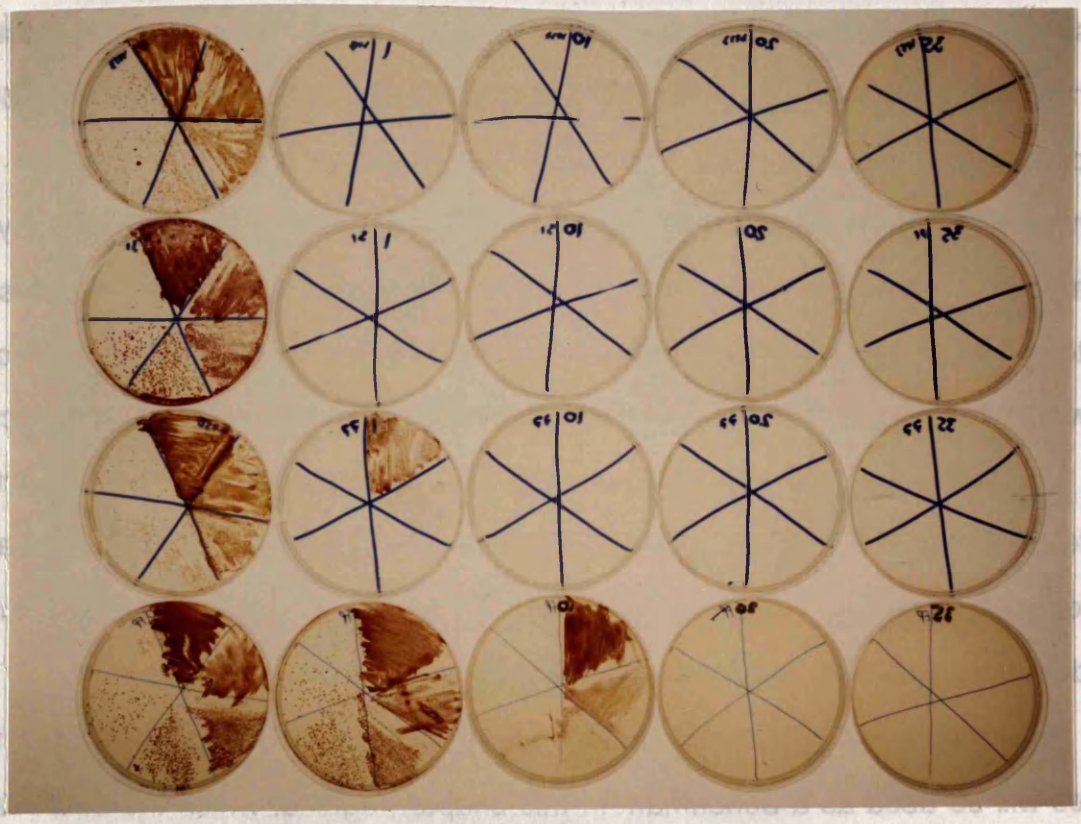


**Figure 6.1a:** Effect of the antibiotic tetracycline (Tc) on the members of the Rhodospirillaceae in this study. This antibiotic binds to the 30S ribosomal subunit thereby inhibiting translocation. From left to right the plates contain 0, 1, 10, 20, 35 µg/ml antibiotic, for clarity the plates containing 50 and 100µg/ml, on which there was 100% killing, have been omitted. The species are, from top to bottom, *Rb. sphaeroides* 8253, *R. rubrum* S1, *Rps. acidophila* 7750, *Rps. palustris* 2.1.6.

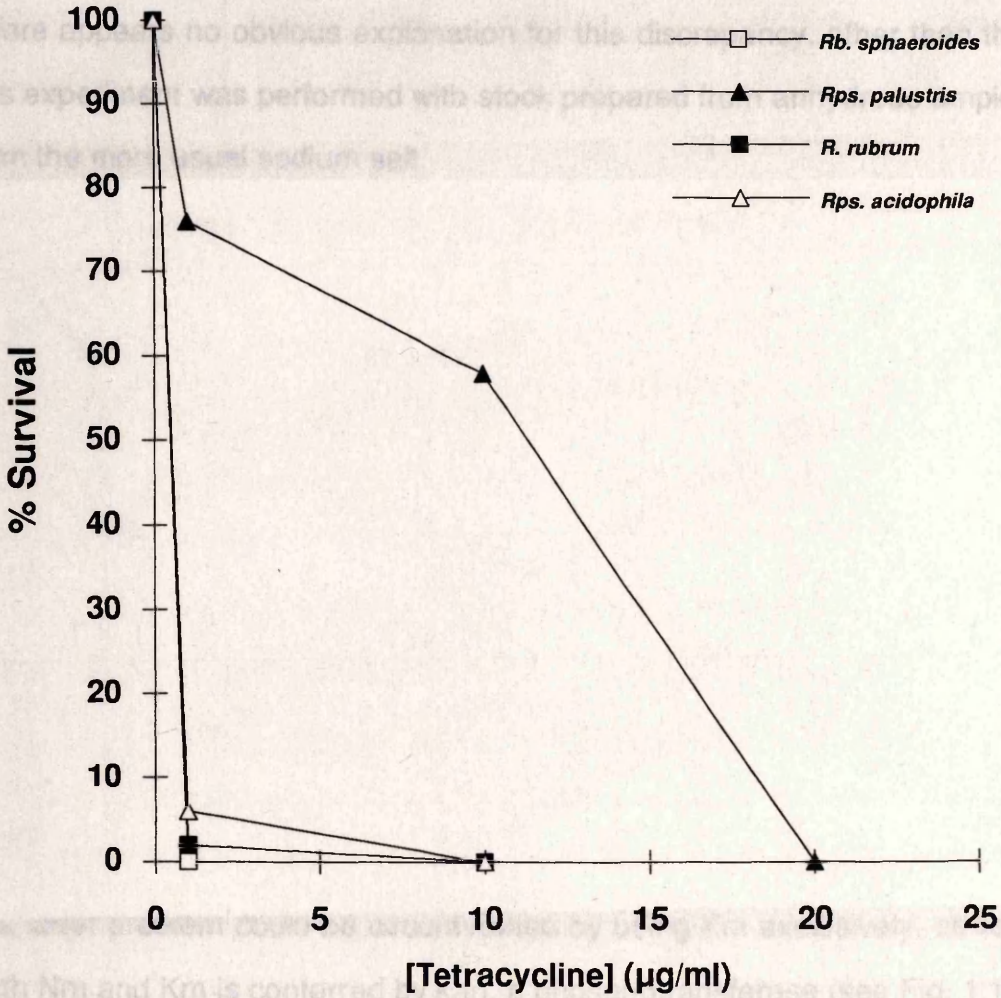



**Figure 6.1b:** Effect of tetracycline on the four Rhodospirillaceae species in this study. This graph was compiled from the previous figure by the procedure described in the Methods (2.4), and illustrates that concentrations of 10µg/ml were sufficient to produce 100% killing for *Rb. sphaeroides* 8253, *R. rubrum* S1 and *Rps acidophila* 7750 whereas 20µg/ml was required for *Rps. palustris* 2.1.6. Equivalent graphs for the other antibiotics are presented in Appendix C.

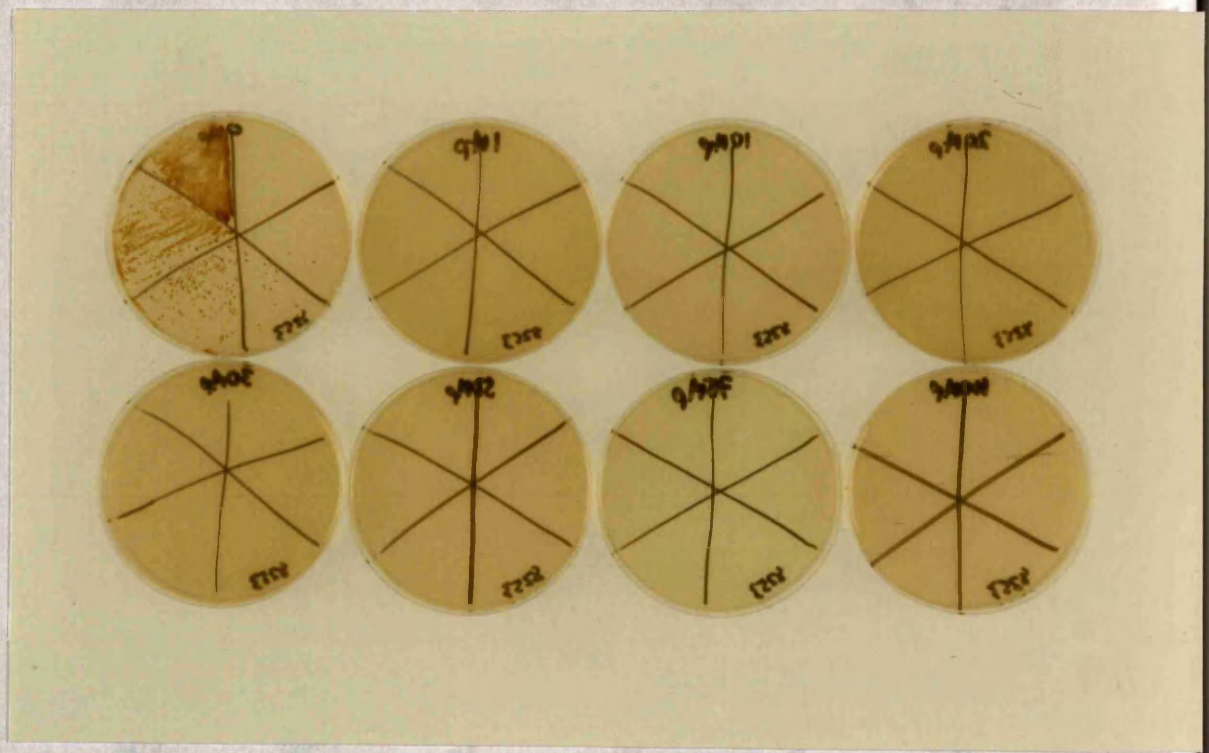


**Figure 6.2:** Effect of ampicillin (Ap) on *Rb. sphaeroides* 8253. This antibiotic belongs to the ß-lactam class that inhibits peptidoglycan biosynthesis. It has previously been reported to be ineffectual with regard to this species, however, in this experiment concentrations >1µg/ml were lethal.

ampicillin (Ap) does not work with this species (Miller and Kaplan, 1978), however, Figure 6.2 indicates that concentrations in this experiment <1µg/ml were effective. There appears no obvious explanation for this discrepancy, other than the fact that this experiment was performed with stock prepared from anhydrous ampicillin rather than the more usual sodium salt.

***R. rubrum***: This species proved susceptible to Ap, Sm, Tc at 10µg/ml and to Km and Nm at 1µg/ml.

***Rps. palustris***: This species demonstrated the greatest range of sensitivity to the antibiotics tested. Ap and Km mediated killing was effective at 10µg/ml, Tc required 20µg/ml, Sm necessitated 100µg/ml and Nm was not considered for the forthcoming experiments as even at 100µg/ml approximately 10% of the cells survived.

***Rps. acidophila***: This species mirrored, albeit to a lesser extent, *Rps. palustris,* Ap, Km and Tc could be used at <10µg/ml, Sm required 35µg/ml and Nm 100µg/ml.

These results showed it was feasible to use in the forthcoming mating experiments the antibiotics, Ap, Km, Nm, Sm and Tc in conjunction with any of the four species under study with the exception of Nm with *Rps. palustris.* Fortunately, this latter problem could be circumvented by using Km exclusively, as resistance to both Nm and Km is conferred by kan, a phosphotransferase (see Fig. 1.15a).

In practice, with a few matings if the minimum antibiotic concentration suggested by these results was used, a few colonies often grew on the control plates. To ensure 100% killing, therefore, and to maintain a strong selection pressure, the final antibiotic concentration selected for the mating experiments was increased. The antibiotic concentration used in the forthcoming matings is outlined for each species in Table 6.1

**Table 6.1 Antibiotic concentration sufficient to achieve 100% killing.**

| Selectable marker. | *Rb. sphaeroides* | *R. rubrum* | *Rps. palustris* | *Rps. acidophila* |
|---|---|---|---|---|
| **Ampicillin** | 50 | 50 | 50 | 50 |
| **Kanamycin** | 30 | 30 | 30 | 30 |
| **Neomycin** | This antibiotic was not used. | | | |
| **Streptomycin** | 50 | 50 | 100 | 50 |
| **Tetracycline** | 10 | 10 | 50 | 30 |

**Numbers refer to the antibiotic concentration given in µg/ml.**

## 6.3 Di-parental matings.

This type of conjugation experiment involves the transfer of a plasmid from *E. coli* (donor) to a Rhodospirillaceae cell (recipient) and the subsequent expression of the marker gene, in this case antibiotic resistance.

### 6.3.1 Matings with Inc-P group plasmid RP1.

RP1 is a 60kb self-transmissible plasmid i.e., it is both $Tra^+$ and $Mob^+$, and carries resistance to Ap, Km and Tc. Although of limited use as a cloning vector due to its size. attempts to produce smaller derivatives were initially hindered by the fact that non-continuous regions are required for replication. The plasmid was already known to be stable in *Rhizobium leguminosarum, Proteus mirabilis* (Jacob *et al.,* 1976) and *Methylophilus methylotrophus* (Windass *et al.,* 1980), and that transfer from *E. coli* to *Rb. sphaeroides* is possible (Miller and Kaplan, 1978) and at very high efficiencies (Hunter, C.N., pers. comm.). This first series of matings was intended to utilise both these facets; broad host range and high efficiency of mobilisation, to establish the feasibility of using conjugation to transfer genes into the species under study. The simple rationale being that if RP1 could not be mobilised successfully, then it would be unlikely that any of the more suitable mobilisable cloning vectors would fare better. The resulting transfer efficiencies for this plasmid are given in Table 6.2. and plates with the *trans*-conjugant *Rb.*

*sphaeroides* cells expressing ampicillin resistance shown in Figure 6.3, in addition to an example of the method by which the transfer efficiency for this mating i.e., *E. coli* DH5[RP1] x *Rb. sphaeroides* 8253 was calculated.

**Table 6.2: Transfer efficiencies obtained for RP1.**

***E. coli* DH5[RP1] x Rhodospirillaceae**

| Selectable Marker | *Rb. sphaeroides* | *R. rubrum* | *Rps. palustris* | *Rps. acidophila* |
|---|---|---|---|---|
| **Ampicillin** | $4.4 \times 10^{-2}$ | $4.9 \times 10^{-2}$ | $2.5 \times 10^{-2}$ | $5.4 \times 10^{-2}$ |
| **Kanamycin** | $3.6 \times 10^{-1}$ | $5.4 \times 10^{-2}$ | $7.8 \times 10^{-2}$ | $1.1 \times 10^{-2}$ |
| **Tetracycline** | $2.2 \times 10^{-1}$ | $6.1 \times 10^{-2}$ | $3.5 \times 10^{-2}$ | $2.9 \times 10^{-4}$ |

These results confirm the broad host range of plasmid RP1 as the plasmid is mobilised across into each recipient species and antibiotic resistance expressed. Figure 6.4 shows the difference in transfer efficiency for *Rps. acidophila* strain 7750 when plated on Tc or Ap.

### 6.3.2 Matings with the Inc Q group plasmid $pNH_2$.

Incompatibility Group Q plasmids such as RSF1010 have been shown to be stable in several species of Rhodospirillaceae. These experiments used a derivative of RSF1010, a 9.45kb $Tc^r$, $Sm^r$ and $Mob^+$ plasmid, $pNH_2$ (Hunter and Turner, 1988). This series of matings use *E. coli* strain Sm10 which has the transfer functions integrated on its chromosome. This type of mating is illustrated schematically in Figure. 1.13. Tc was used in preference to Sm for *Rps. palustris* and *Rps. acidophila* because of these species relatively high tolerance to this antibiotic. The transfer efficiencies obtained are presented in Table. 6.3.

These results show that $pNH_2$ does not transfer as well to the other species as to *Rb. sphaeroides* 8253, although, for *R. rubrum* S1 the efficiency is still very high.

Suspension of *E. coli* strain DH5 containing plasmid RP1.

True $OD_{600}$ : DH5[RP1] = 30.0

= $2.2 \times 10^{10}$ cells per ml, (from graph in Appendix B).

= $1.1 \times 10^{8}$ cells in 5µl.

Log-phase chemoheterotrophic culture of *Rb. sphaeroides*.

True $OD_{650}$ : 8253 = 0.384

= $5.1 \times 10^{8}$ cells per ml, (from graph in Appendix B).

Ten fold dilution = $5.1 \times 10^{7}$ cells per ml

= $2.3 \times 10^{6}$ cells per 45µl.

In order to give a recipient:donor ratio of 20:1 the donor cells were diluted by:

$$\frac{1.1 \times 10^{8}}{(2.3 \times 10^{6} \times 20)} = 2.4$$

The cell mixture was spotted onto a dried LB-plate, evaporated to dryness and incubated for exactly six-hours at 37°C. The cells were then scraped off with a wire inoculating loop, resuspended in 1ml of minimal media and serial dilutions made, prior to plating on plates, plus or minus antibiotic. From the resultant colonies the highest dilution that could accurately be counted was taken. The transfer efficiency, i.e., the number of drug-resistant *trans*-conjugants obtained relative to the number of recipients recovered, was calculated as follows:

20µl of the $10^{-2}$ dilution of mated cells from antibiotic plates gave 51 colonies.

20µl of the $10^{-3}$ dilution of mated cells from non-antibiotic plates gave 111 and 118 colonies, i.e., 115 average.

Antibiotic plates = 51 x 100 x (1000/20) = $2.55 \times 10^{5}$.

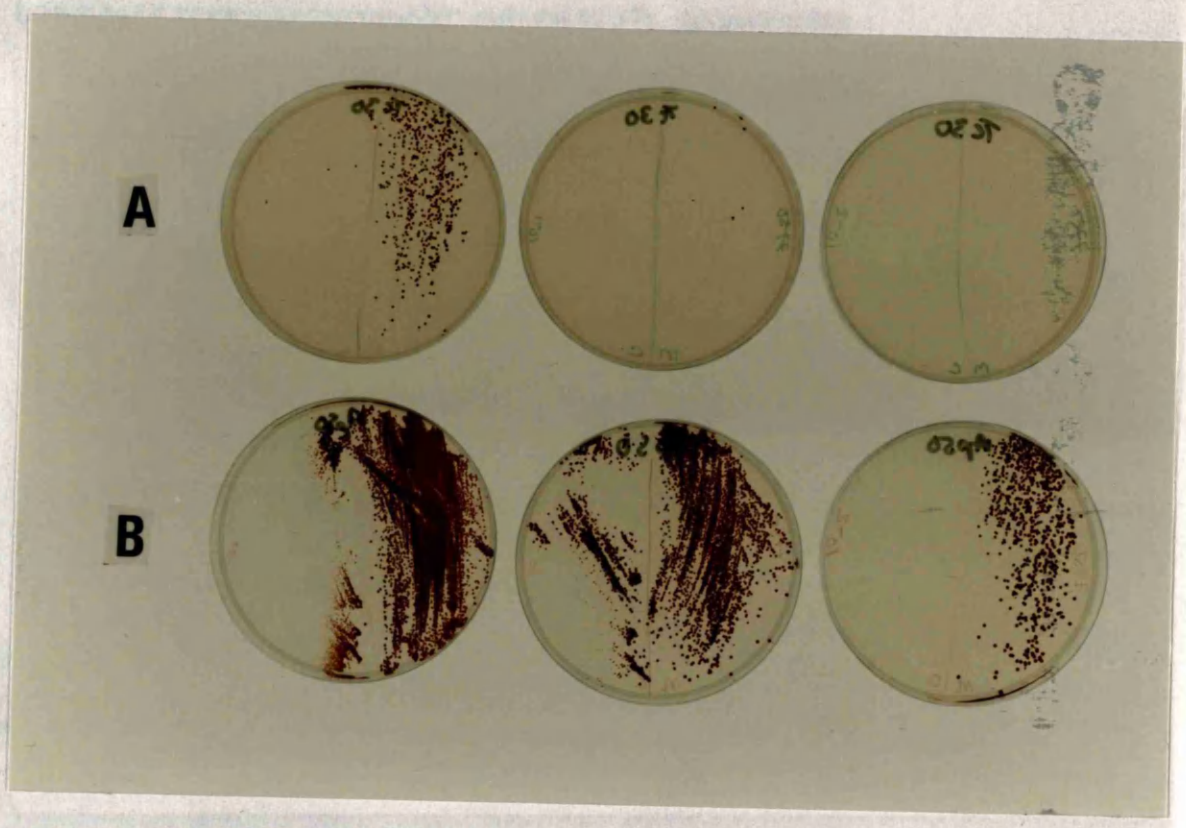
Non-antibiotic plates = 115 x 1000 x (1000/20) = $5.75 \times 10^{6}$

Therefore, the transfer efficiency is: $\frac{2.55 \times 10^{5}}{5.75 \times 10^{6}} = 4.4 \times 10^{-2}$

or, for every 1000 *Rb. sphaeroides* cells that were recovered from the mating procedure, 44 had successfully acquired and were expressing antibiotic resistance.

***E. coli* DH5[RP1] x *Rb. sphaeroides* strain 8253.**

**Figure 6.3:** The left-hand plate does not contain antibiotic and is used to estimate the number of cells recovered after the mating. The right-hand plate contains ampicillin, the left-hand side of which was plated with unmated cells that are all killed; the right-hand side was plated with the mating mix and the number of surviving *trans*-conjugants counted. The calculation for the transfer efficiency for this mating is given opposite.




***E. coli* DH5[RP1] x *Rps. acidophila* 7750**

**Figure 6.4:** *Trans*-conjugant 7750 cells were plated on tetracycline (row A) or ampicillin (row B), This experiment illustrates the variability in expression of the resistance markers *in vivo* resulting in different transfer efficiencies. Each plate is divided into two halves; the left-hand side has been plated with an approximately equivalent number of unmated cells to ensure selective pressure is being applied and as expected all are killed. The right-hand side, has been plated with trans-conjugant, mated cells, containing RP1 conferring $Ap^r$ and $Tc^r$, therefore, these colonies are able to grow on the selective media.

**Table 6.3: Transfer efficiencies obtained for $pNH_2$.**

***E. coli* Sm10[$NH_2$].x Rhodospirillaceae**

| Selectable Marker | *Rb. sphaeroides* | *R. rubrum* | *Rps. palustris* | *Rps. acidophila* |
|---|---|---|---|---|
| Streptomycin | $4.4x10^{-1}$ | $8.7x10^{-2}$ | na. | na. |
| Tetracycline | na. | na | $5.6x10^{-4}$ | $7.8x10^{-4}$ |

**na.= not attempted.**

### 6.3.4 Matings with an improved Inc-P group plasmid.

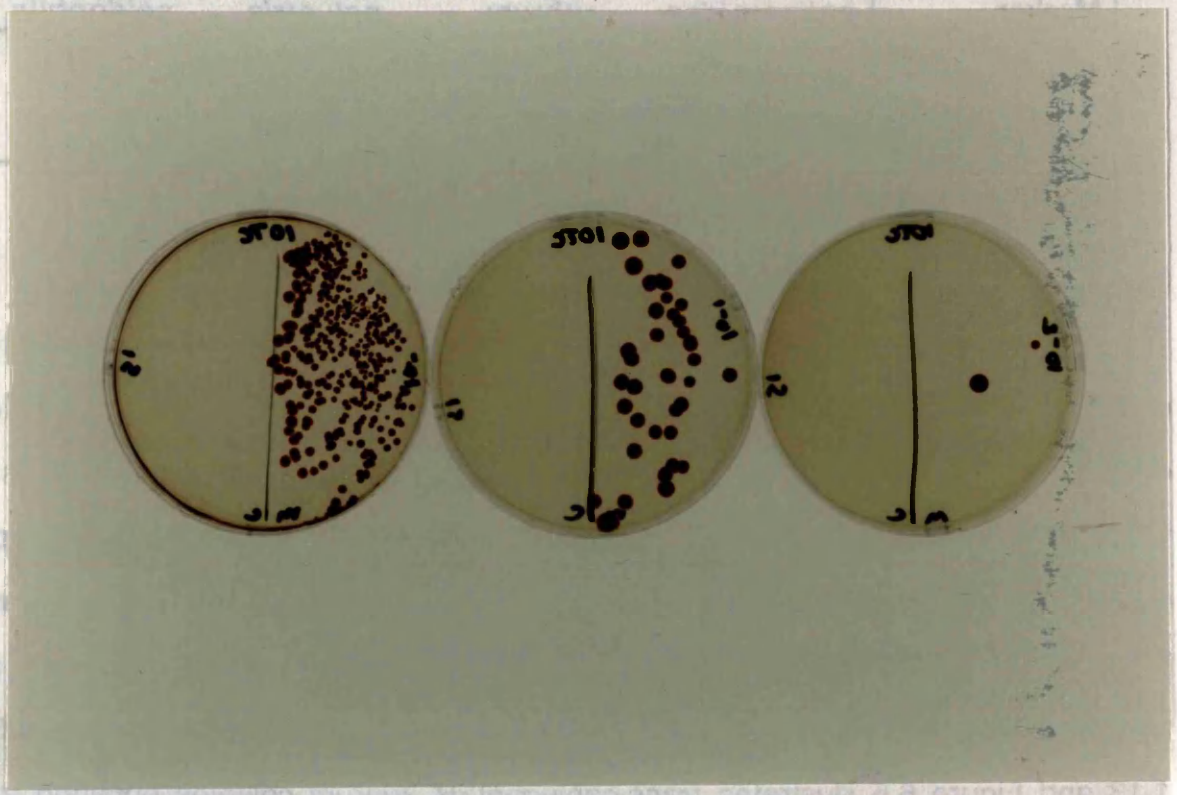
The naturally occuring R-factor RP1 was the starting point for the construction of small derivatives which contained polylinker sites and lac Z facilitating efficient cloning and screening of recombinants (Ditta *et. al.*, 1985). Thus, the series of vectors produced are much more suitable for molecular biology than either of those detailed above. The plasmid construct that was used for this series of matings was pRK404, a 10.6kb $Tc^r$, $Mob^+$ plasmid. and the transfer efficiencies obtained are presented in Table 6.4. A mating with this plasmid is illustrated schematically in Fig. 1.13 and Figure 6.5 illustrates *trans*-conjugant *R. rubrum* colonies obtained after expressing resistance to this antibiotic.

**Table 6.4: Transfer efficiencies obtained for pRK404.**

***E. coli* Sm10[RK404] x Rhodospirillaceae.**

| Selectable Marker | *Rb. sphaeroides* | *R. rubrum* | *Rps. palustris* | *Rps. acidophila* |
|---|---|---|---|---|
| Tetracycline | $2.1x10^{-4}$ | $1.6x10^{-3}$ | $2.6x10^{-4}$ | No colonies. |

This set of results demonstrates that pRK404 (and its derivatives) are able to be mobilised into three of the species under study, notwithstanding the fact that the transfer efficiencies obtained are 2-3 orders of magnitude less than those obtained



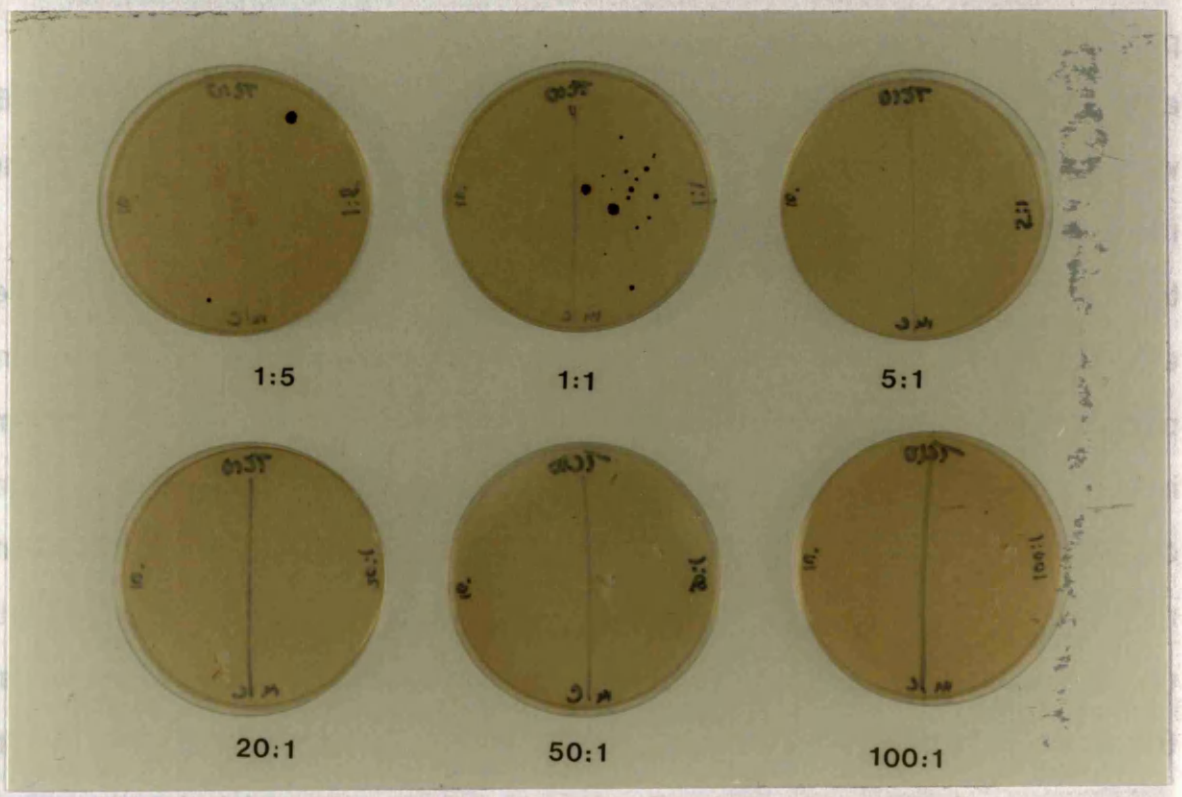
***E. coli* Sm10[RK404] x *R. rubrum* S1**

**Figure 6.5:** *Trans*-conjugant *R. rubrum* S1[RK404] cells are illustrated plated on tetracycline.

for the Inc-Q plasmid $pHN_2$. As stated previously (2.5) the aim of this chapter was to establish the feasibility of setting up a convenient and reproducible system to transfer genes of choice into Rhodospirillaceae and not to rigorously and exhaustively test every variable for its effect on the transfer efficiency. In order to produce *Rps. acidophila trans*-conjugant colonies, however, it became necessary to alter parameters which were previously fixed. The results presented in Table 6.4 were attained with a recipient:donor ratio of 20:1; it was selected as it is used routinely in other laboratories with *Rb. sphaeroides,* facilitating comparison of transfer efficiencies, and not because it would necessarily result in the highest efficiency. Indeed within the context of this study it would neither be desirable nor practical to attempt to optimise this parameter, for all four species, with each plasmid in every experiment. Nevertheless, in this case as no *Rps. acidophila* resistant colonies were attained, it was necessary to evaluate a range of recipient:donor ratios, in order to introduce pRK404 into this species. It was hoped that within the range of ratios tried, one would optimise the contacts between the *E. coli* pilli and the recipient bacteria, resulting in transfer of the plasmid and expression of the marker gene. In the end a ratio of 1:1 proved most successful giving a transfer efficiency, albeit rather low, of $9.4 \times 10^{-6}$. This mating is illustrated in Figure 6.6 where the effect of varying this ratio is readily apparent.

## 6.4 Tri-parental matings.

Having established that the separate conjugative transfer of RP1 and $pNH_2$ into those Rhodospirillaceae species under study is possible, and that both are stably maintained, the next set of experiments were designed to test the ability of RP1 to mobilise $pNH_2$ tri-parentally i.e., two different donors are involved. This experiment could be contemplated because RP1 and $pNH_2$ belong to different incompatibility groups. An incompatibility group can be defined as a set of plasmids whose members are unable to coexist in the same bacterial cell. The presence of a member of one incompatibility group does not directly affect the survival of a



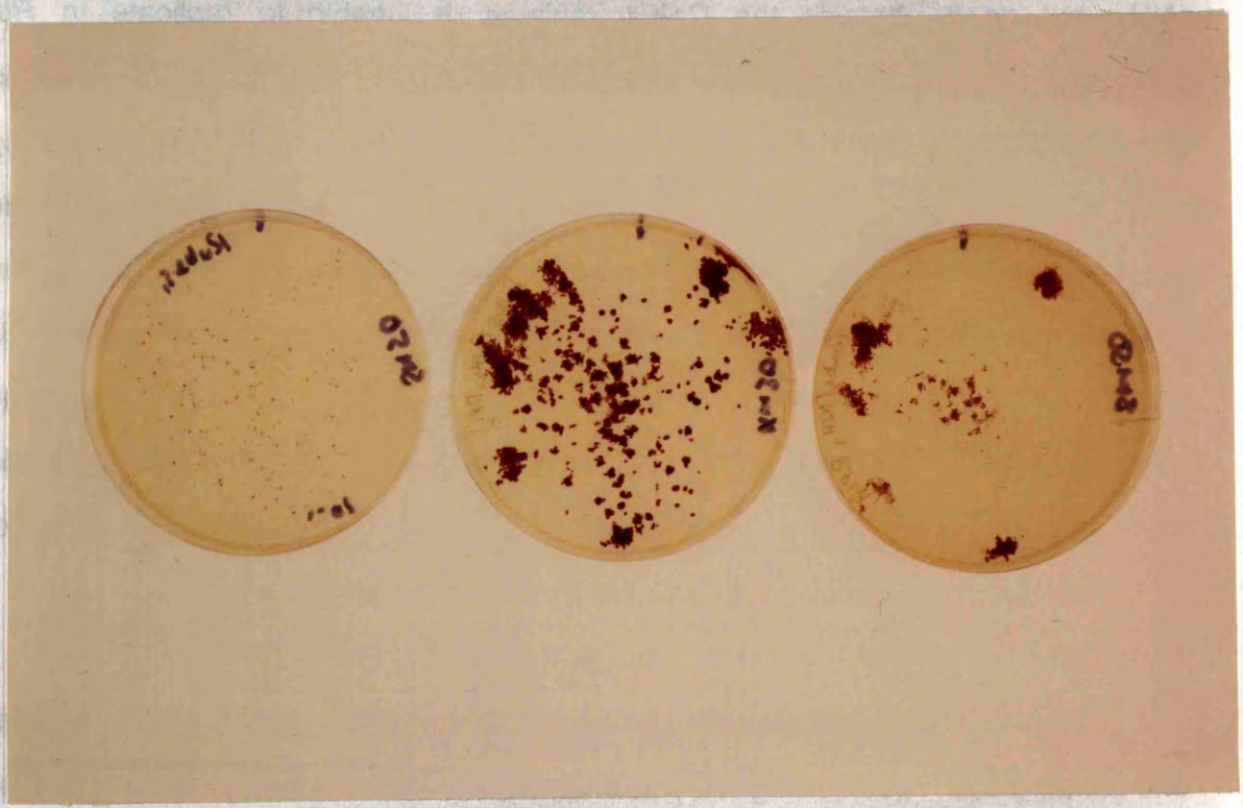

***E. coli* Sm10[RK404] x *Rps. acidophila* 7750**

**Figure 6.6:** This mating was initially unsuccessful at a recipient:donor ratio of 20:1. Various different ratios, therefore, were tried the most successful being 1:1. In this particular case the concentration of tetracycline was lowered to the minimum value suggested in Figure 6.1 i.e., 10µg/ml to ensure that the selection pressure in the previous unsuccessful mating (Table 6.4) was not too strong.

plasmid belonging to a different group. Thus only one replicon of a given incompatibility group can be maintained in the bacterium, it does not interact with replicons of other incompatibility groups (although in limiting conditions they may compete for physical space within the cell). In addition the self-transmissible Inc-P plasmid pRK2013 (Ditta *et al.,* 1985) was used in place of RP1: it can mobilise $pNH_2$, but as it contains the ColE1 replicon, is unable to replicate in *Rb. sphaeroides.* To date the best characterised replicon is that of the ColE1 plasmid from *E. coli* (Tomizawa and Itoh, 1981), which requires for its functioning, among other things, a 555bp RNA transcript primer, the enzyme RNase H, the rop gene product and the synthesis of RNA I. Because the concerted action of these different components is neccessary, it is perhaps not surprising that the ColE1 replicon is only functional in the Enterobacteriaceae, of which *E. coli* is a member. Advantage can be made of this narrow host range because it has the effect that any plasmid which bears this replicon is non-viable outwith this genera. One such plasmid, pRK2013, is thus able to act as a 'suicide' donor of transfer functions.

### 6.4.1 **Tri-parental matings involving tra donated by RP1.**

Each Rhodospirillaceae species in this study was mated with two donors; one contained RP1 (conferring $Km^r$) as a donor of transfer functions and the cloning vector $pNH_2$ (conferring $Sm^r$). This type of mating is illustrated schematically in Figure 1.14a. Immediately after the mating procedure the cells were plated with Sm selection, enabling the calculation of transfer efficiencies in a similar manner to Fig. 6.3b. The colonies were then replica plated onto Km to ensure that RP1 had also been transferred from the donor. The efficiency of transfer for the $Sm^r$ marker is shown in Table 6.5. An example of this type of mating for *R. rubrum* S1 is shown in Figure 6.7. The mated Rhodospirillaceae cell contains both RP1 and $pNH_2$, possible as both plasmids belong to different incompatibility groups. Furthermore, as each plasmid is able to be propagated within the recipient, in this case S1, it is able to survive under both selection regimes.



***E. coli* DH5[RP1] x *E. coli* DH5[$NH_2$] x *R. rubrum* S1.**

**Figure 6.7:** The *trans*-conjugants were plated initially on Sm (left) and then replica plated on Km (centre) and Sm (right). RP1, conferring $Km^r$, is maintained in *R. rubrum* S1 so that cells plated on media containing this antibiotic are viable.

**Table 6.5 Tri-parental matings involving tra donated by RP1**

***E. coli* DH5[RP1] x *E. coli* DH5[$NH_2$] x Rhodospirillaceae**

| | *Rb. sphaeroides* | *R. rubrum* | *Rps. palustris* | *Rps. acidophila* |
|---|---|---|---|---|
| **Transfer of $Sm^r$ from $pNH_2$** | **$5.4x10^{-3}$** | **$2.3x10^{-3}$** | **ns** | **$8.1x10^{-4}$** |

**ns = not successful for reasons outlined in the text.**

Although the experiments previously described suggested that Sm at 100µg/ml would be sufficient to give 100% killing of *Rps. palustris* 2.1.6, in practice there always appeared a few colonies on the control plate. Thus, although superficially successful, it was not possible to conclude categorically that the $Sm^r$ colonies were the result of having acquired plasmid $pNH_2$, and not from a high basal level of natural resistance. No results, therefore, are entered for this mating: the $Tc^r$ marker of $pNH_2$ could not be used because of the inability to distinguish between $pNH_2$ resistance and that conferred by RP1.

### 6.4.2 Tri-parental matings involving tra donated by pRK2013.

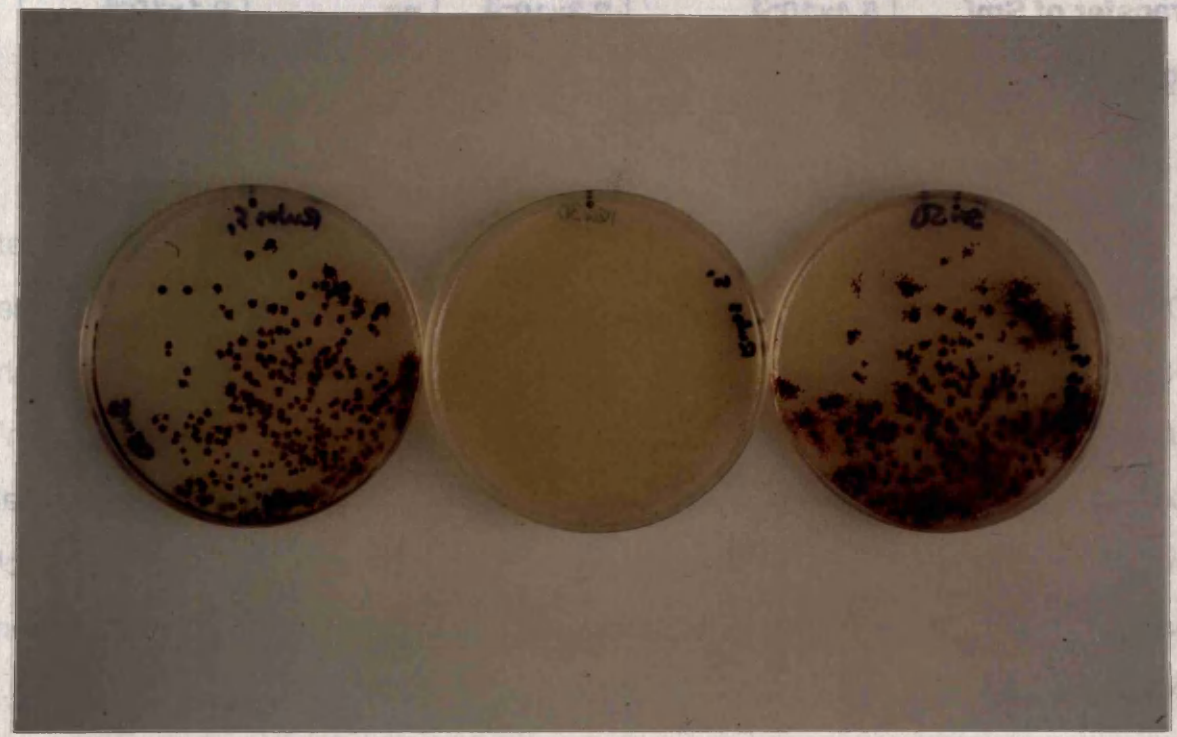
This type of mating is illustrated schematically in Figure 1.14b and the efficiency of transfer is given in Table 6.6.

**Table 6.6 Tri-parental matings involving tra donated by pRK2013**

***E. coli* DH5[RK2013] x *E. coli* DH5[$NH_2$] x Rhodospirillaceae**

| | *Rb. sphaeroides* | *R. rubrum* | *Rps. palustris* | *Rps. acidophila* |
|---|---|---|---|---|
| **Transfer of $Sm^r$ from $pNH_2$** | **$5.8x10^{-3}$** | **$7.5x10^{-3}$** | **$2.2x10^{-4}$** | **$8.5x10^{-5}$** |

Once again the cells were plated initially on Sm and then replica plated on Km, and then back to Sm. Thus, should the cells die, this death is due to Km and



***E. coli* DH5[RK2073] x *E. coli* DH5[$NH_2$] x *R. rubrum* S1**

**Figure 6.8:** The *trans*-conjugant cells were plated as in Figure 6.7 As pRK2013 contains the ColE1 replicon, this plasmid is unable to replicate in S1, thus, the cells are killed when plated on the centre plate which contains Km.

not to the plating procedure. The mating for *R. rubrum* S1 is illustrated in Figure 6.8. In this situation both plasmids are mobilised into S1, and the cells are viable when plated on Sm but not on Km. pRK2013 contains the ColE1 replicon and is rapidly lost from the cells along with the kanamycin resistance that it confers, therefore, when plated on this antibiotic the cells are killed. This result was mirrored for the other three species.

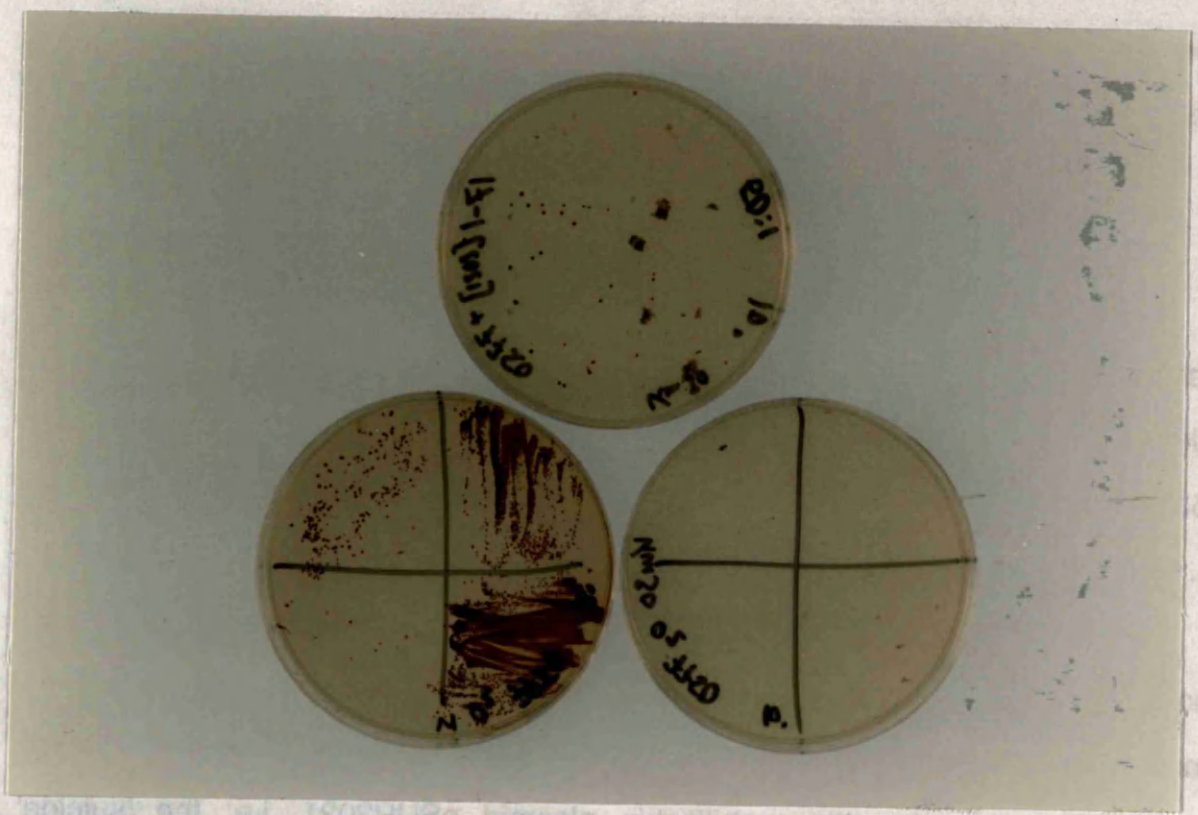
## 6.5 Transposon mutagenesis of Rhodospirillaceae.

These experiments were conceived with the straight-forward aim of extending the applicability of random transposon mutagenesis to the three species under discussion. A wealth of data has already been published for *Rb. sphaeroides* 8253 (see e.g. Hunter, 1988) and *Rb. capsulatus* (Kaufmann *et al.,* 1984).

### 6.5.1 Mating with the Tn5 containing plasmid pSUP2021.

Transposon Tn5 (see Fig 1.15a) has been used most often for the apparently random construction of auxotrophs or other mutants, therefore, Tn5 was the transposon of choice for the following matings and is delivered into the Rhodospirillaceae using the mobilisable plasmid pSUP2021, i.e, the 'suicide' plasmid pSUP202 carrying Tn5 (Simon *et al.,* 1983). Thus, once the conjugation process has finished and the plasmid harbouring Tn5 has been transferred into the recipient cell, the antibiotic resistance conferred by the transposon will be expressed and the Rhodospirillaceae cell will be viable. The vector plasmid is dependent upon the non-functional ColE1 replicon for its replication, with the result that it is lost from the cells. A schematic illustration of this experiment was presented in Figure 1.15b.

As with the other di-parental matings described previously, *E. coli* strain 17-1 (donor) [not strain Sm10] containing the mobilisable 'suicide' plasmid pSUP2021 was mixed with Rhodospirillaceae (recipient) in the ratio 20:1. After mating the cells were resuspended and plated, as described in the Methods (2.5.1). The frequency



***E. coli* 17-1[SUP2021] x *Rps. acidophila* 7750.**

**Figure 6.9:** The colonies on the top plate are *trans*-conjugants from the above mating and are presumed to carry the Tn5 transposon. The bottom left plate are cells from the chemoheterotrophic culture used for the mating, grown photoheterotrophically, to ensure there had been no adventitious contamination. The bottom right plate is the same culture plated on Km to ensure the antibiotic is effective and all the cells plated are killed.

which $Km^r$ colonies appear is given by transfer efficiency x transposition frequency and the results presented in Table 6.4.

**Table 6.4: Mating to introduce the Tn5-bearing plasmid, pSUP2021 into the recipient cell.**

***E. coli* 17-1[2021] x Rhodospirillaceae**

| Selectable Marker | *Rb. sphaeroides* | *R. rubrum* | *Rps. palustris* | *Rps. acidophila* |
|---|---|---|---|---|
| Kanamycin | $2.9x10^{-6}$ | $3.9x10^{-7}$ | $3.1x10^{-6}$ | $1.1x10^{-6}$ |

The appearance of $Km^r$ *Rps. acidophila* 7750 colonies after mating with this plasmid is shown in Figure 6.9. These results are suggestive but not conclusive proof that transposition has occurred. If the ColE1 replicon was able to be maintained in one or all of these species the selection pressure inside such a cell on the transposon to 'transpose' would be less, consequently the frequency of $Km^r$ colonies would approach the transfer frequently (for 8253 this is of the order $10^{-2}$; Hunter, C. N., pers. comm.), and the transposon would be propagated through vegetative plasmid propagation instead of genomic replication. A further possibility is that part or all of pSUP2021 could have been integrated into the genome through a recombination event. It was necessary, therefore, to verify that transposition into the genome had occurred with the concomitant loss of the vector plasmid, pSUP2021. In order to do this the following experiments were undertaken.

### 6.5.2 Verification of pSUP2021 'suicide'.

The above matings were repeated for each species (in practice this was only necessary for S1) a sufficient number of times in order to generate enough $Km^r$ colonies to be toothpicked onto a circular Hybond-N filter in a 5x5 array. Similar arrays were made of unmated cells, the rationale being that these colonies do not contain Tn5 and so act as a negative control. After photoheterotrophic colony regeneration the arrays were lysed and the DNA fixed by baking. Finally, the filters

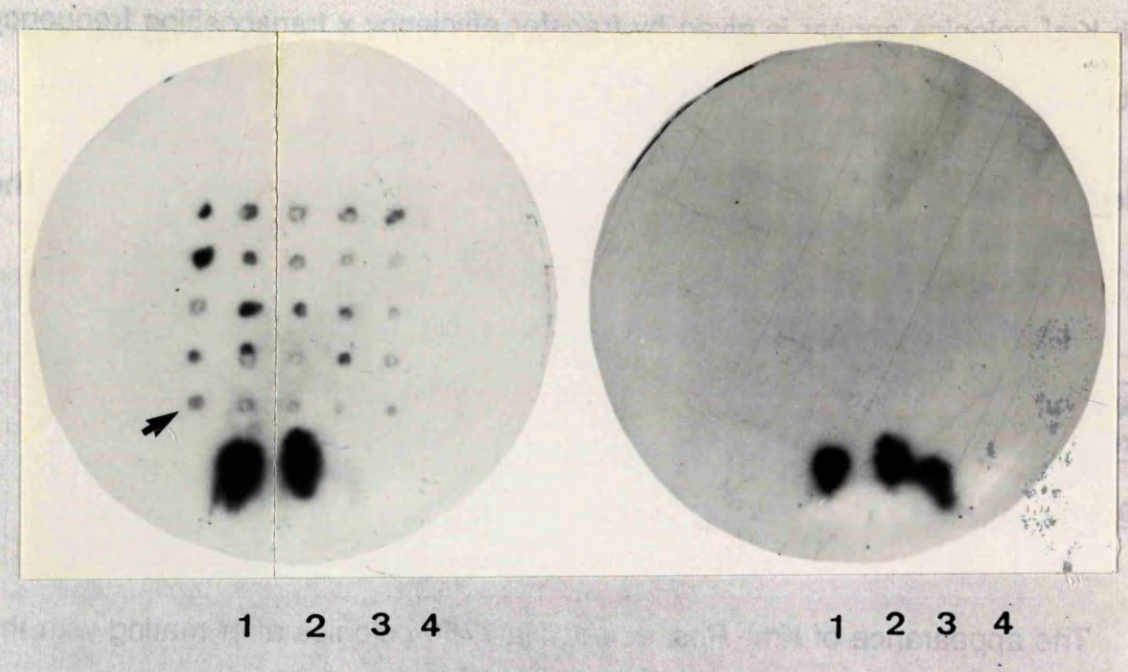



**Figure 6.10:** Autorads from the arrays of putative Tn5 containing 7750 colonies. The left autorad was probed with pKan2 and the right with pSUP202. The numbers designate control DNAs and the arrow identifies the the colony from which genomic DNA was isolated to confirm the presence of Tn5.

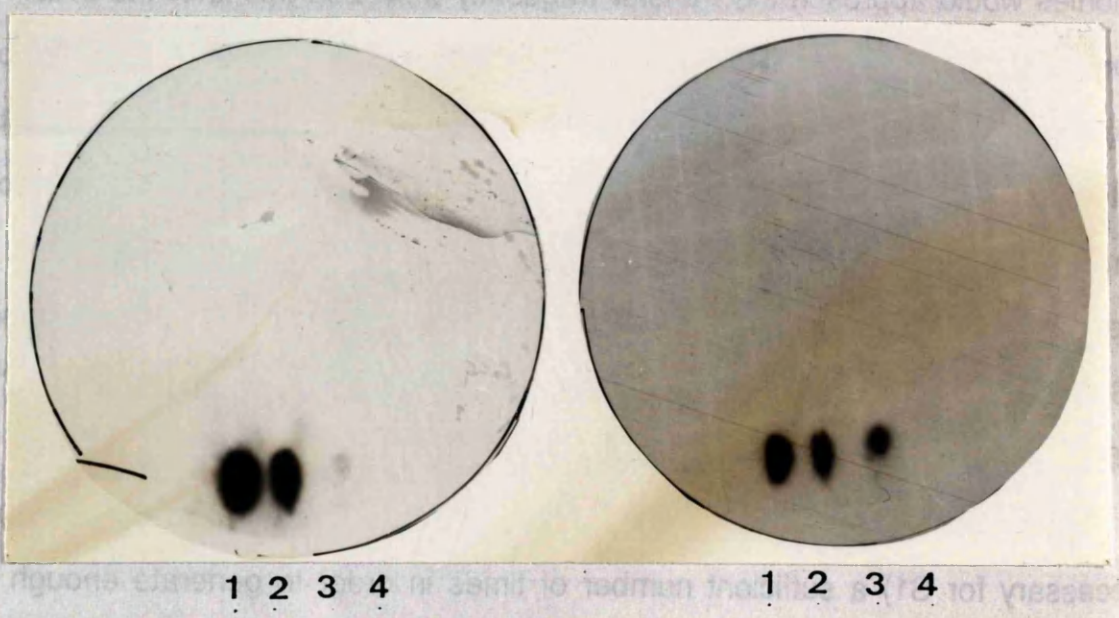



**Figure 6.11:** Autorads from the wild type i.e., unmated, 7750 array, probed as above. As expected, no signal was observed as the cells contained neither Tn5 nor the vector plasmid, pSUP2021.

were washed to remove obtrusive cell debris. The precise protocol for this procedure is outlined in the Methods (2.8). Filters were probed sequentially with pKan2 and with pSUP202, the resultant autoradiographs for *Rps. acidophila* are presented in Figures. 6.10 and 6.11.

From Figure 6.10 it is apparent that every colony contains at least one copy of Tn5, however the hybridisation signal in each case is rather variable. This is possibly due to a combination of factors:

(1) *Rps. acidophila* has in the past proved extremely difficult to lyse (MacKenzie, 1990). It is likely that this protocol even with the NaOH step elongated to 30min, although adequate for *Rb. sphaeroides,* is not optimum for 7750 rendering inconsistent lysis.

(2) Assuming Tn5 transposes randomly into the genome, a certain amount of these events will be fatal to the cell, others will interrupt genes which under the prevalent growth conditions are not being expressed and as such will have no effect. However, a certain amount will mutate a gene necessary (but not critical) for growth and as a consequence will debilitate the cell. A pertinent example would be transposition into the puc operon necessitating photoheterotrophic growth to rely on light harvesting from the B890/RC conjugate only. Thus the overall effect will be to generate a population in which a proportion of the cells have different growth rates, in the 5x5 array this will mean a faster growing colony when lysed has a greater total number of transposons that will hybridise.

(3) After transposition from the plasmid into the genome it is possible for another or indeed more transposition events to occur. This again has the effect of varying the total number of transposons in any given colony within the array. A corollary to this point is that transposons can be thought of as 'selfish' in the Dawkins sense (Dawkins, 1976): they must transpose to be propagated, yet too many transposition events would prove lethal to the cell; overall a balance is struck in which the transposon is carried into the next generation (Cohen and Shapiro, 1980).

After pKan2 was washed off, the filters were re-hybridised with pSUP202 (Fig. 6.11), no signal was detected suggestive of the fact that this plasmid is not replicated in *Rps. acidophila* i.e., the ColE1 replicon is indeed non-functional. This same conclusion was reached with the other three species under study. As expected the unmated cells gave no signal to either pKan2 or pSUP202.

Four DNA control spots hybridised as expected; pKan2 and pSUP2021 hybridised to the pKan2 probe, pSUP202 hybridised only to the pSUP202 probe and neither probe hybridised with M13 mp19 RF DNA.

### 6.5.3 **Verification of transposition.**

The next step was to confirm that Tn5 had transposed into the bacterial genome. One colony from each master plate, marked with an arrow in Fig 6.10, 8 in total (4 species: mated and control) were grown up in liquid culture and genomic DNA isolated. The four control DNA samples were then digested with a number of restriction endonucleases which were selected because their sites are known within Tn5 (Figure 1.15a) and therefore yield identifiable fragments when the transposon is cleaved with that enzyme. The gel for wild type (WT) *Rps. acidophila* strain 7750 (i.e., unmated) DNA is presented in Figure. 6.12. The next step involved digesting the DNAs and Southern Blotting onto Hybond-N filters prior to hybridising with the pKan2 probe, described fully in the Methods (2.6.8/9/10). The resultant autoradiograph for 7750 is shown in Figure 6.13.

As expected WT DNA produced no signal in lane 1 as it does not contain Tn5. The DNA in lanes 2-5 was prepared from the putative Tn5 containing arrowed colony in Fig 6.10 and is designated AG772. Lane 2 DNA has been cleaved with EcoRI; this enzyme does not cut within Tn5 resulting in a single band with a molecular weight around 15.8kb i.e., the transposon (5.7kb) has inserted into a 10.1kb EcoRI fragment. Lane 3 DNA has been cleaved with BamHI; Tn5 contains only one BamHI site at position 3070, therefore, digestion with this enzyme produces two bands corresponding to hybridisation upstream and downstream from this site. The DNA in lanes 4 and 5 was intended as controls; lane 4 is cut with

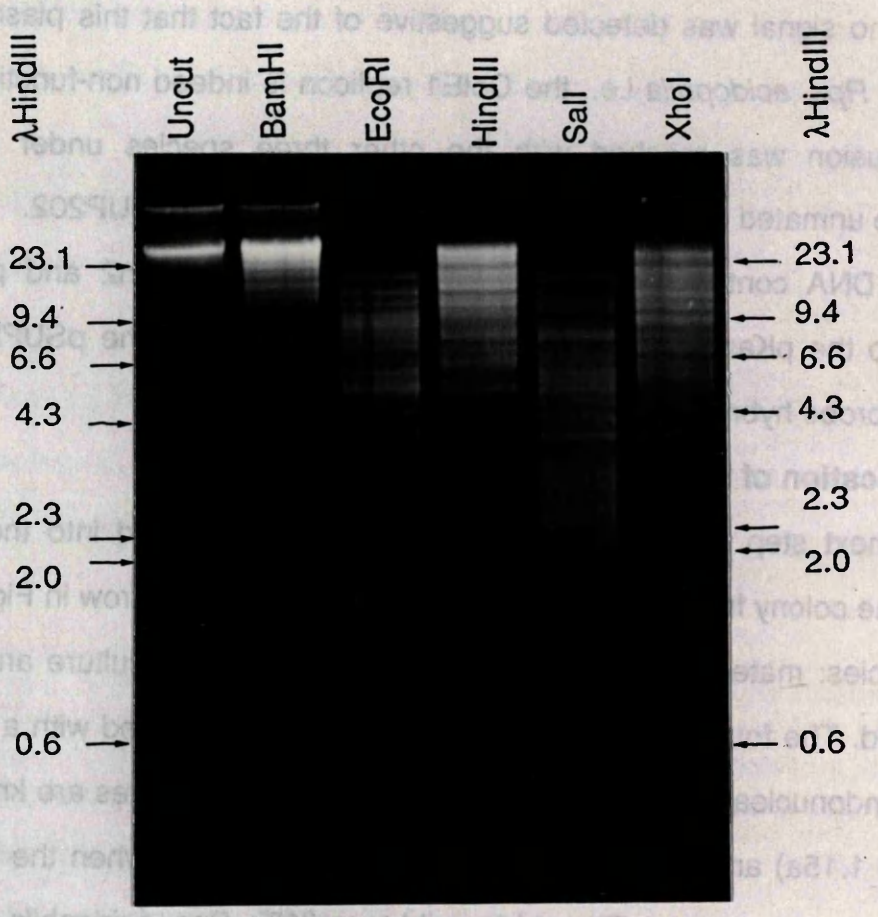



**Figure 6.12:** Restriction digest of wild type 7750 genomic DNA. Lane 1 contained 1µg DNA, the other lanes 3µg.

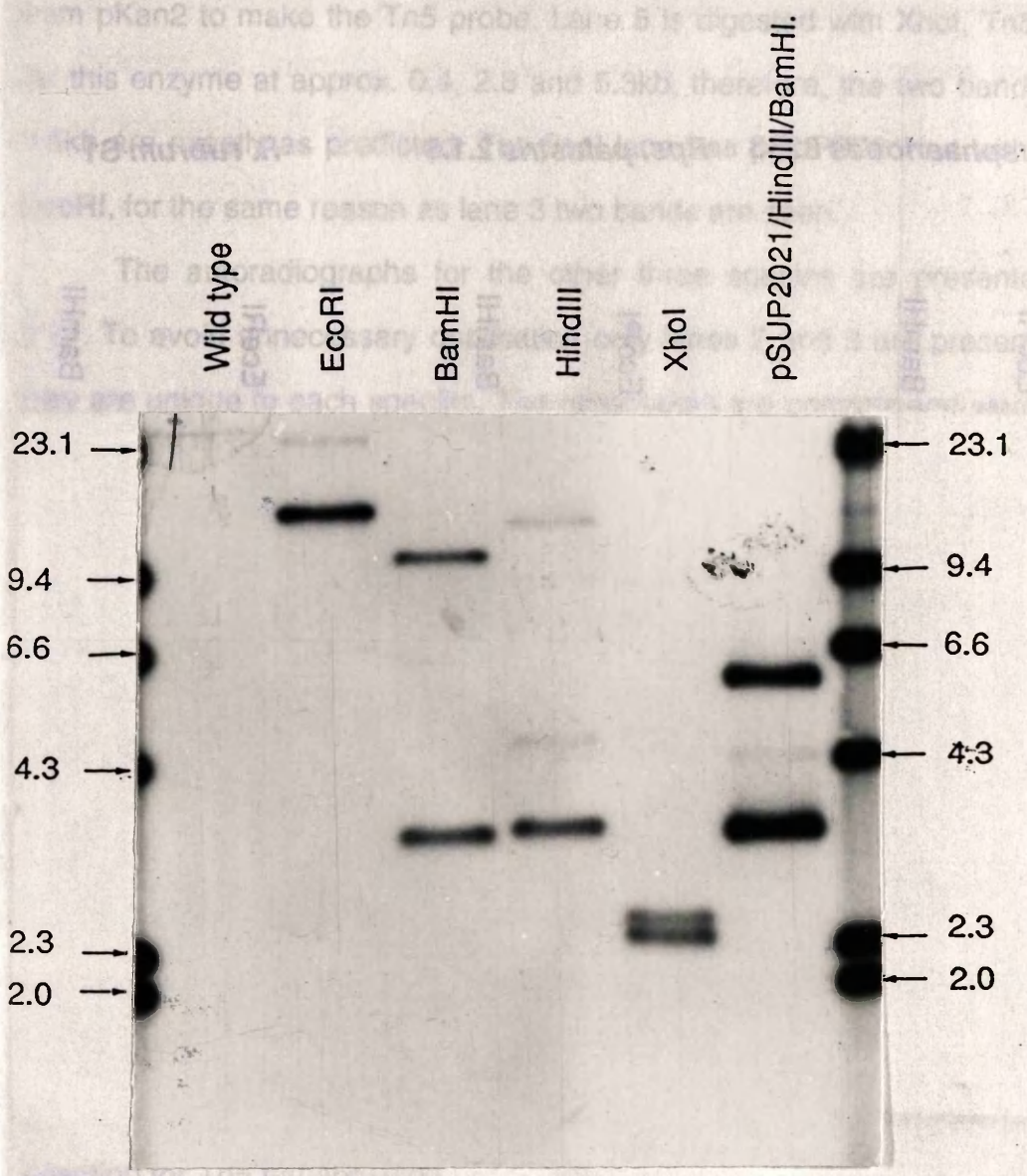



**Figure 6.13:** Autoradiograph obtained after probing *Rps. acidophila* 7750 genomic DNA (mutant AG772) with pKan2.It is apparent that this mutant has Tn5 integrated into its genome. A full explanation of the banding pattern is given in the text.

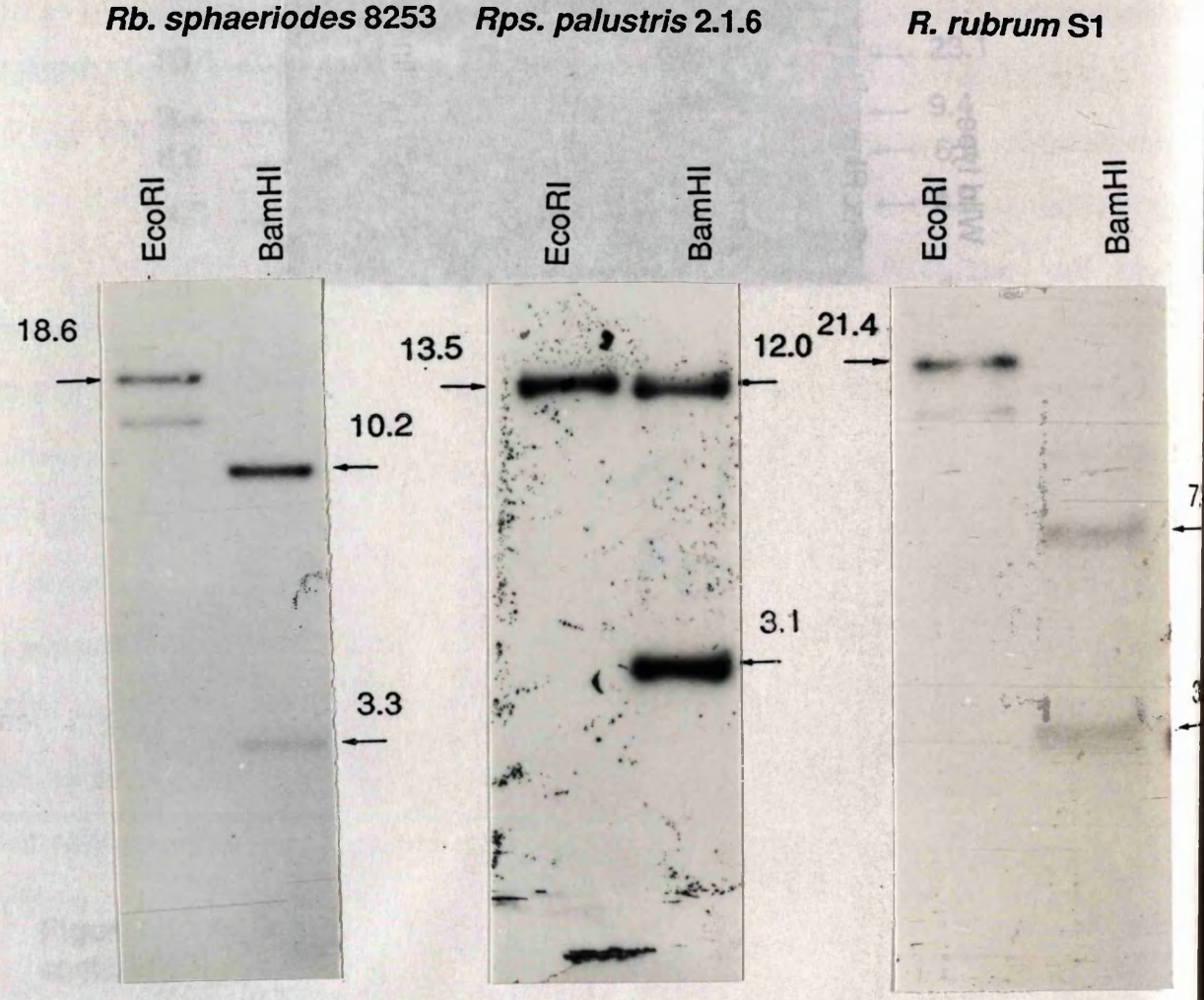



**Figure 6.14:** These autoradiographs show, after probing with pKan2, the insertion of Tn5 into the genomes of the other species in this study. The control lanes which were identical to the previous figure, have been omitted for conciseness. The transposon has inserted into EcoRI fragments of 12.9, 7.8, and 15.7kb for *Rb. sphaeroides* 8253, *Rps. palustris* 2.1.6 and *R. rubrum* S1 respectively. In a similar manner to *Rps. acidophila* 7750 in the previous figure, S1 and 8253 show evidence of a secondary transposition event.

HindIII and excises a band at 3.3kb. This is the middle part of the transposon between the HindIII sites at 1195 and 4580 and is the precise fragment excised from pKan2 to make the Tn5 probe. Lane 5 is digested with XhoI, Tn5 has 3 sites for this enzyme at approx. 0.4, 2.8 and 5.3kb, therefore, the two bands at 2.4 and 2.5kb are exactly as predicted. The final lane has pSUP2021 cut with BamHI and EcoRI, for the same reason as lane 3 two bands are seen.

The autoradiographs for the other three species are presented in Figure 6.14. To avoid unnecessary duplication only lanes 2 and 3 are presented because they are unique to each species. The other lanes are controls and were identical to those in Fig. 6.13.

## 6.6 **Conclusions.**

This chapter was intended to assess the viability of extending the conjugation based system of Simon *et al.,* (1983), to members of the Rhodospirillaceae. Overall the results presented indicate that this system is indeed applicable to the other species studied, providing a reliable and reproducible method for introducing cloned DNA and transposons into the recipient species, all of which were chosen because of their intrinsically interesting peripheral antenna complements. One minor proviso is that care is taken with the antibiotic selection used e.g., $Tc^r$ is preferable to $Sm^r$ in certain cases with Inc-Q plasmids, similarly $Km^r$ is preferable to $Nm^r$ when selecting for Tn5 transposition.

Both Inc-P and Inc-Q derived plasmids are stably maintained in *R. rubrum* S1, *Rps. palustris* 2.1.6 and *Rps. acidophila* 7750, and can be introduced either through di- or tri-parental matings. However, the expression of the tetracycline resistance gene appears poor in *Rps. acidophila* 7750, with the result that the transfer efficiencies were consistently less for this species.

Using this system to introduce transposons into these species was also successful. The experiment in this chapter proved that Tn5 had transposed into the genome of the recipient and the vector plasmid lost from the cell. This has important

significance because standard molecular techniques involving transposons will be able to be employed. The mating conditions, however, for *R. rubrum* will also require modification to increase the production of mutants, although other work (Sockett, E., pers. comm.) has confirmed that this species appears relatively unamenable to Tn5 mutagenesis.

# Chapter Seven

## Sequencing of a *Rps. acidophila* strain 7050 peripheral antenna multigene cluster.

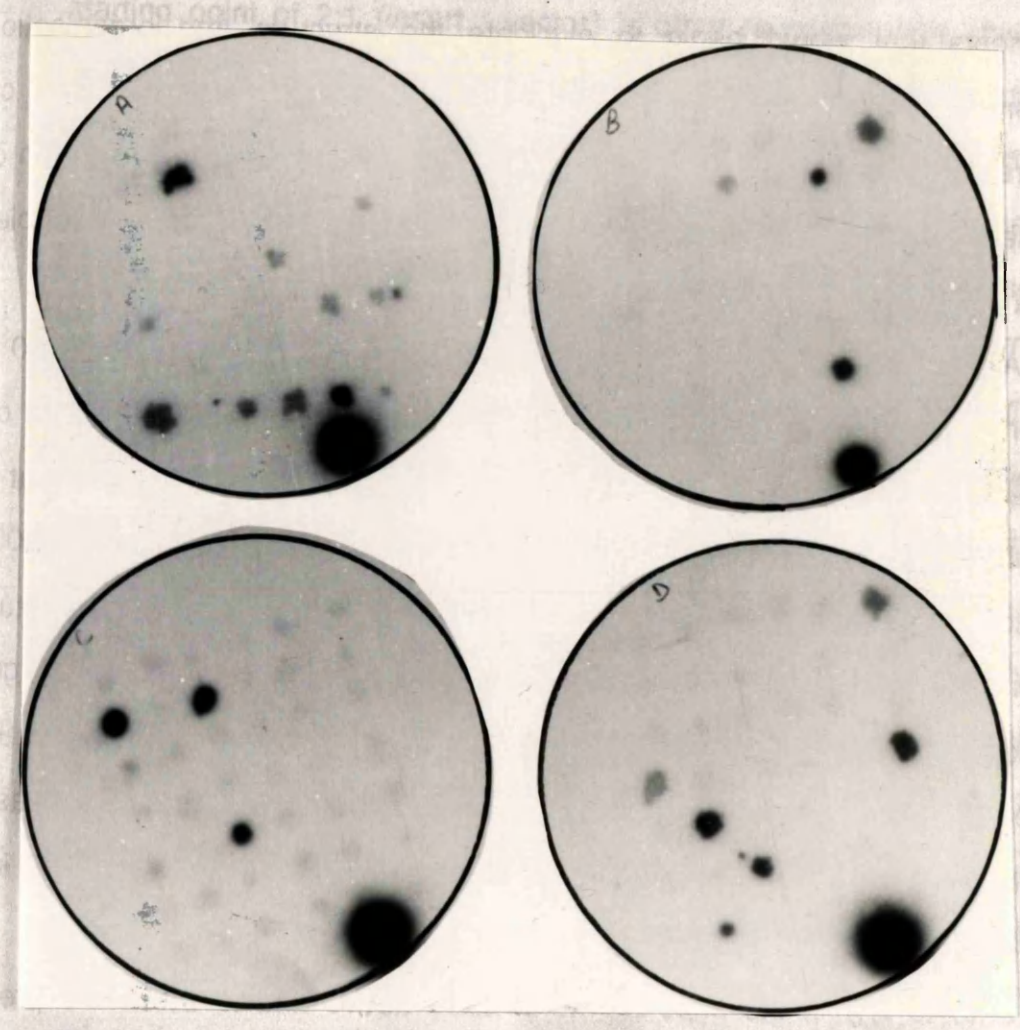



**Figure 7.1:** White transformants were toothpicked onto a 6 x 7 array, lysed, and probed with pLHIISB18. Most colonies do not produce a signal as their insert does not contain antenna complex genes, therefore, positives were grown up and their inserts characterised. Colonies were named depending on their position in the array, e.g. B52 is from filter B, fifth coloumn, second row up. Two control DNAs were fixed on the filter; (1) pBluescript, below and left of the array, as expected no signal was detected, and (2) pLHIISB18, below and right of the array, which gave a strong signal.

## 7.1 Introduction.

The previous chapter has established that transfer of cloned DNA into *Rps. acidophila* is feasible. It is now possible to contemplate molecular gene experiments that would begin to elucidate the structure and organisation peripheral antenna complex genes and their regulation. The aim, therefore, of t work presented in this chapter was to identify a suitable piece of DNA which co be cloned into a mobilisable vector and hence, introduced into a suitable recipient effect a response.

An EMBL3 library has been constructed from *Rps. acidophila* 7050 DN (M$^{ac}$Kenzie, 1991) and a preliminary attempt made at characterising some of t peripheral genes present in this strain. From this work it became evident that the are multiple copies of β/α gene pairs encoding the peripheral complexes B800-8 and B800-820 present in the genome, and that DNA from one of the clones studie λ6, contains one of these β/α gene pairs on an approx. 2.1kb SalI fragme Depending on the precise position of the gene pair on this fragment it should sufficiently large to contain any upstream regulatory regions as well as a downstream sequences analogous to pucC (Tichy *et al.,* 1991) [see 1.8] in *R. capsulatus* and *Rb. sphaeroides.*

Prior to any of the experiments detailed above and to determine precis what structural genes, open reading frames (ORFs), regulatory elements, etc., t this fragment contained, it was necessary to fully characterise this fragment throu DNA sequencing.

## 7.2 Cloning of *Rps. acidophila* 7050 peripheral antenna complex genes.

As a prerequisite to sequencing, the first step was to shotgun clone t hybridising (to pLHIISB18) 2.1kb SalI fragment from λ6 into the SalI site in multiple cloning site of pBluescript® II SK+ (Stratagene Ltd., Cambridge, UK). T vector is a 2961bp phagemid, derived from pUC19 which allows, amongst ot

applications blue/white colony selection, creation of ExoIII deletions and double stranded sequencing: all functions necessary in the course of this chapter.

For ligation, the ideal ratio of insert to vector DNA is variable, however, a starting point of 2:1 (insert : vector) is often recommended, this is measured in available pmol ends per µg DNA. For the calculation it was assumed that the mol. wt. of λ6 DNA was 50kb and the actual ligation was carried out with Bacteriophage T4 DNA Ligase (1WeißU) at 14°C overnight (for a definition of one Weiß Unit, see Sambrook *et al.,* 1989. pp 5.62). No attempt was made to reduce self-ligation of the vector by treating with calf intestinal alkaline phosphatase (CIAP). The following day 1µl of the ligation mix was used to transform competent *E. coli* XL-1Blue cells, plated on LB containing X-gal and IPTG, and incubated overnight to allow for blue/white colony selection. This resulted in an approximate blue/white ratio of 10:1. White colonies were then toothpicked onto four Hybond-N nylon filters in 6x7 arrays (168 whites in total) for screening. This number seemed initially over-cautious, however, in the event screening this large number proved fortuitous. The colonies were lysed, fixed and probed with pLHIISB18: of the 168 whites probed 24 gave a positive signal, the resultant autoradiograph is shown in Figure 7.1. The twenty strongest hybridising colonies were minipreped to ensure the insert was of the expected size i.e., 2.1kb. These plasmids were cut with SalI, run on a 1% agarose gel, and reprobed with pLHIISB18. The gels and autoradiographs are presented in Figure 7.2a and 7.2b respectively. Close scrutiny reveals that only eleven of the twenty have the expected 2.1kb insert, with or without others. For the remaining nine it is unclear as to why these colonies hybridised so strongly in Fig. 7.1 but gave no signal in Fig 7.2. Even with the subsequent knowledge revealed by sequencing that the antenna genes are not contained solely on the 2.1kb fragment, it is difficult to explain why, e.g., colony D67 which has a 2.2kb insert produced a signal in Fig 7.1. Colony B52, with B56 as backup, were cut with EcoRI to ensure that the plasmid size was approx. 5.1kb i.e., multiple ligation of the 2.1kb fragment into the cloning site had not occurred (gel not shown).

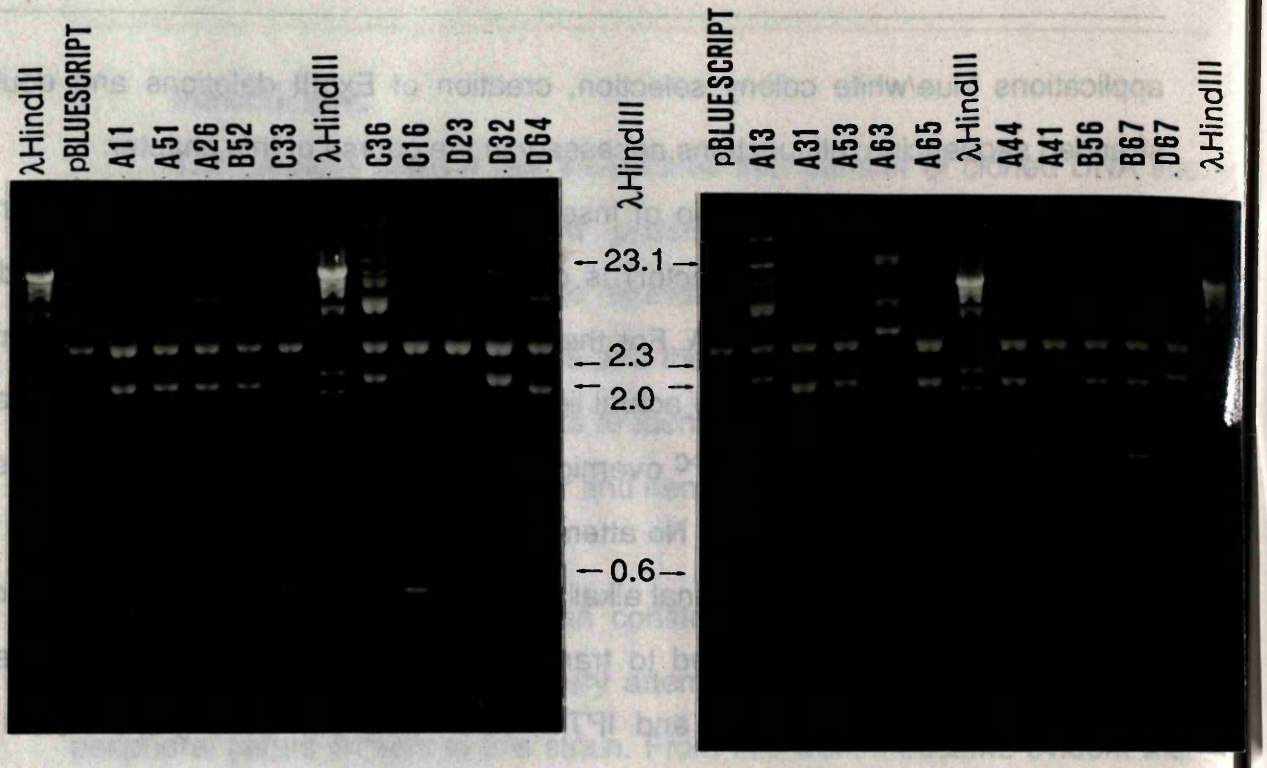
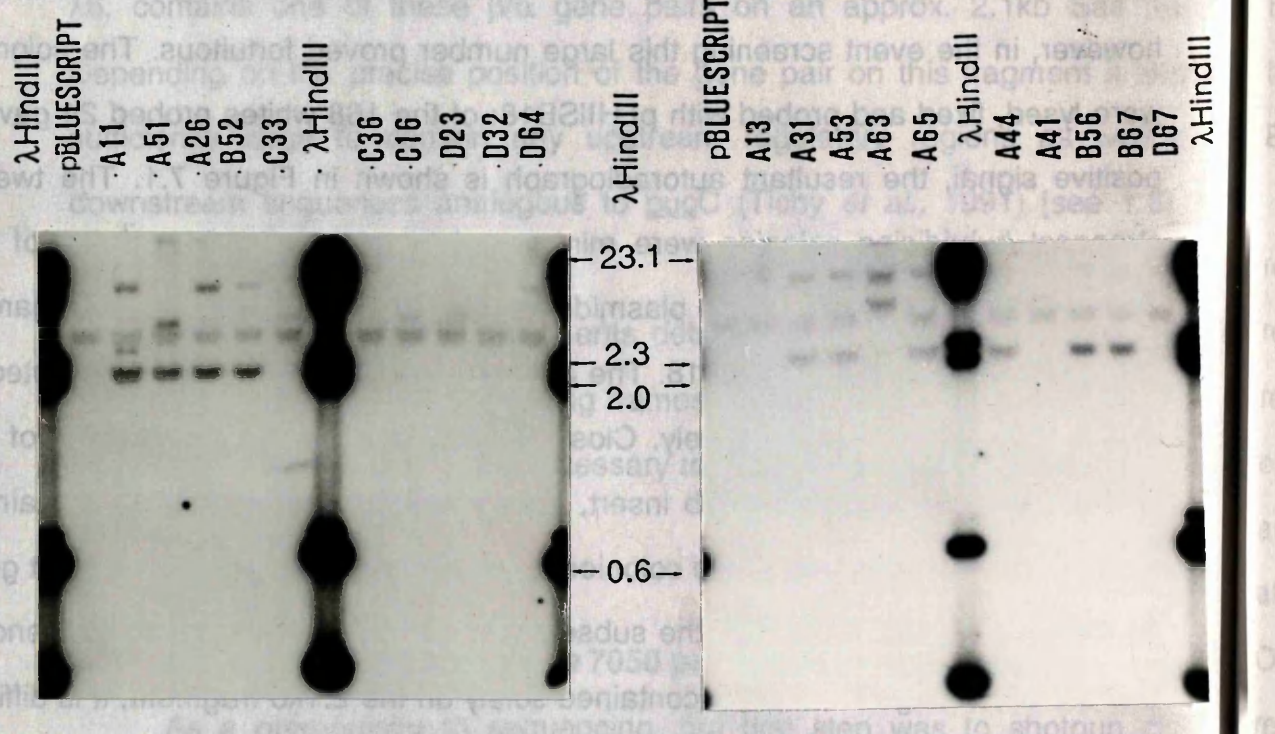



**Figure 7.2: (a)** Positives identified from colony hybridisation were minipreped and the plasmids cut with SalI to excise their inserts. It can be seen that in many of the plasmids multiple ligation has ocurred.




**(b)** The above gels were Southern blotted and probed with pLHIISB18 in order to verify that the 2.1kb SalI insert contained antenna complex genes.

From these results the colony designated B52 was selected for sequencing, by way of nomenclature the vector plasmid (pBluescript) plus insert (2.1kb hybridising fragment [to pLHIISB18] from λ6) was termed pB52.

## 7.3 Sequencing of the pB52 2.1kb insert.

Several methods have now been developed to generate a set of nested deletions that lack progressively more nucleotides from one end or the other of the target DNA. The method chosen in this particular instance was to use the enzyme Exonuclease III (ExoIII) for the following reasons. This enzyme catalyses the stepwise removal of 5'-mononucleotides from recessed or blunt 3'-hydroxyl termini of double stranded DNA; protruding 3' termini are completely resistant to the activity of the enzyme. This property of ExoIII is valuable in that it has been used to develop a protocol that allows construction of unidirectional deletions without subcloning the target DNA. To create unidirectional deletion mutants, the double stranded DNA of a recombinant plasmid is digested with two restriction enzymes whose sites of cleavage both lie between one end of the target DNA and the primer binding site. The enzyme that cleaves nearer the target sequences must generate a blunt end or a recessed 3' terminus; the other enzyme must generate a 3- or 4-nucleotide protruding 3' terminus. Because only one end of the resulting linear DNA is susceptible to ExoIII, digestion proceeds unidirectionally away from the site of cleavage into the target DNA sequence. The exposed single strands are removed by digestion with nuclease S1, and the DNA is then recircularised.

As the insert to be sequenced has been cloned into the SalI site of the pBluescript polylinker, examination of the remaining sites reveals that between the SalI site and the T7 primer binding site there are restriction sites for the enzymes KpnI and XhoI. These enzymes cleave DNA to leave the following termini.

XhoI $^{5'}C$ / $_{3'}GAGCT_{5'}$ KpnI $^{5'}GGTAC\ ^{3'}$ / $_{3'}C$

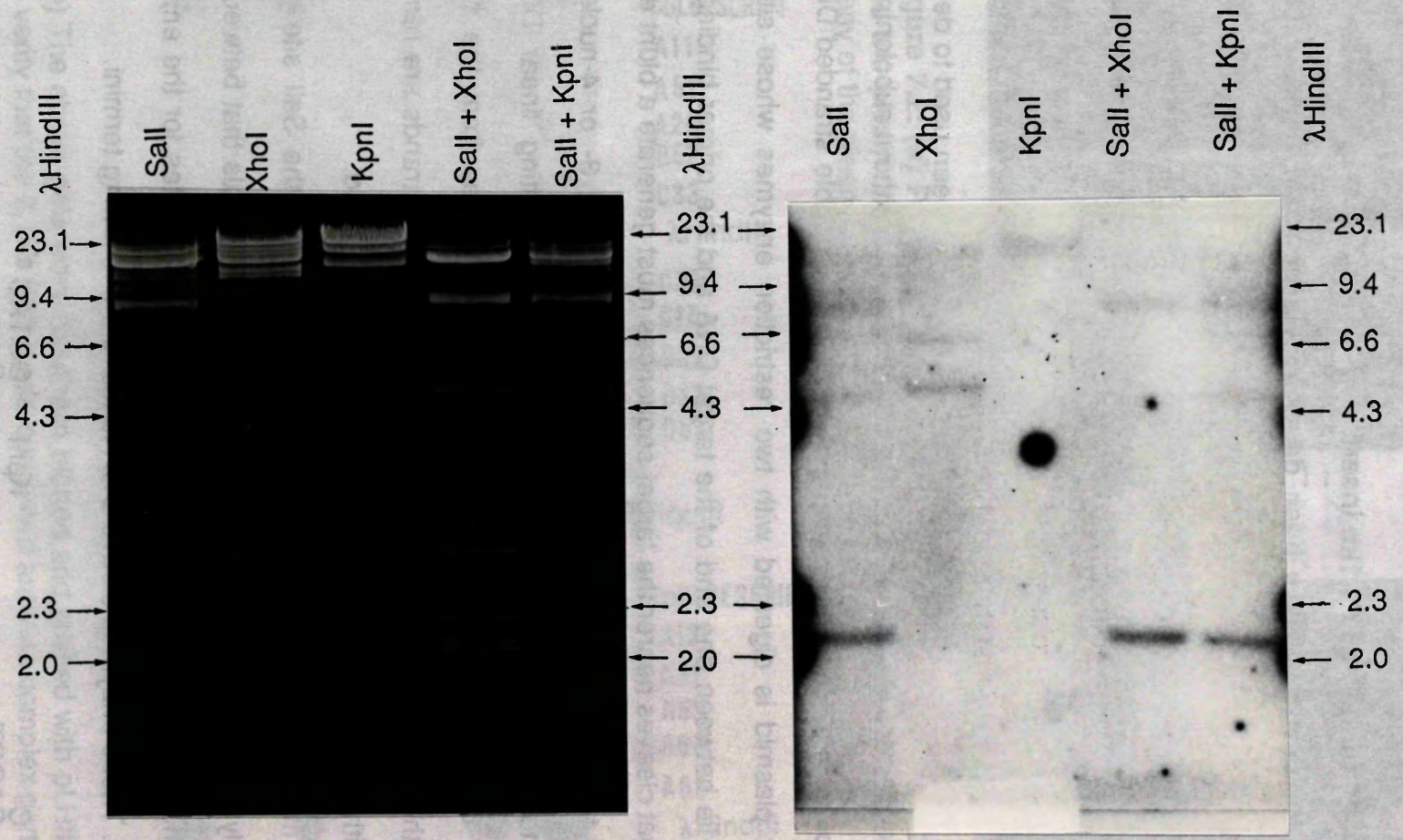



**Figure 7.3:** This gel and susequent autorad prove that there are no internal KpnI or XhoI sites within the hybridising 2.1kb fragment. λ6 DNA was cut with the enzymes shown, it is apparent that the hybridising band remains intact whether it is cut with SalI, SalI + KpnI or SalI + XhoI. It became evident from this experiment that the 2.1kb fragment was in fact a doublet and that only one hybridised to pLHIISB18.

Thus it is apparent that these two enzymes fulfil the above requirements: XhoI by creating a recessed 3' terminus adjacent to the target site and KpnI by creating a 3' overhang adjacent to the primer site thus preventing ExoIII digestion at this site. However there is one self-evident condition that must be met before these enzymes can be employed, namely there must be no XhoI or KpnI sites within the target sequence. That this is in fact the case is proved in Figure 7.3, where λ6 has been digested by SalI, XhoI, KpnI, SalI+XhoI and SalI+KpnI. Digestion by SalI excises the 2.1kb band that has been cloned into pBluescript, XhoI and KpnI individual digests produce as expected different restriction patterns. When the DNA is digested with SalI and XhoI together, SalI excises the 2.1kb fragment but there is no further digestion with XhoI, indicating that there is no site for this enzyme in the target DNA. Precisely the same result can be observed in the lane for SalI+KpnI. The conclusion, therefore, was drawn that there are no XhoI or KpnI sites within the target DNA, this was confirmed when the gel was probed with pLHIISB18, thus these two enzymes fulfil the requirements to create a nested set of deletions using ExoIII.

10μg of pB52 DNA was digested with XhoI then KpnI, phenol extracted, and resuspended in 1 x ExoIII Buffer. 200Units of ExoIII were added and aliquots removed every 30s into nuclease S1. This amount of ExoIII is one third more than recommended in the standard protocol (2.6.11), the extra enzyme was added to ensure all available ends had started enzymatic digestion at time 0 i.e., no lag had arisen whereby some enzyme was searching for an 'end' to digest whilst others had already begun digestion. This ensures that the deletions are uniform at time 0. Once the S1 digestion had been completed for all time points, 1μl aliquots were removed from each and run on an agarose gel, this can be seen in Figure 7.4. After fill-in with Klenow and blunt-end ligation, each time point was transformed separately into XL-1Blue, whereupon three colonies from each were minipreped and their plasmids sized. This allowed the selection of one clone from each time point that was smaller than its counterpart in the previous timepoint by

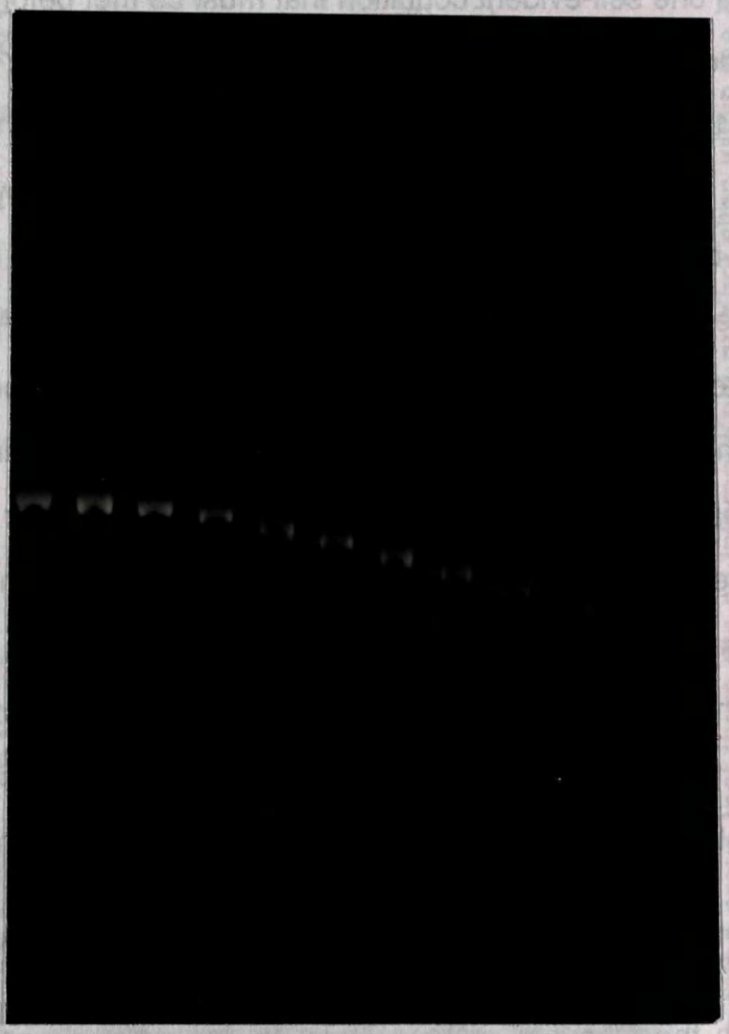


**Figure 7.4:** Gel illustrating the progressive digestion of linearised pB52 by ExoIII from the recessed 3'-terminus after treatment with S1 nuclease. Each timepoint differs from the preceeding one by 30s. Unfortunately due to an error there are no molecular weight markers associated with this figure. (The vector plasmid plus insert at time 0 are approx. 5.1kb).

approximately 100-300bp; sequencing is facilitated because the clones form an overlapping series. The selected colony from each time point (designated clone A for time 0 i.e., before any ExoIII was added i.e., contains undigested insert, clone B for 30s, up to clone I for 240s), was maxipreped to yield high quantity, high quality template for sequencing.

Prior to this however an attempt was made to localise the ß/α gene pair on this fragment. The plasmid DNA selected from each timepoint i.e., clones A to I, was run out on a gel and probed with pLHIISB18, the result is presented in Figure 7.5. It can be seen that there is a strong hybridisation signal from all the clones up until clone H, whereupon the signal drops dramatically. From this it can be inferred that the sequences removed by ExoIII prior to clone H do not contain the genes for the light harvesting polypeptides and by clone I the enzyme has digested most of the genes, hence, the very weak signal attributed to this clone. Thus the end of the β/α pair can be placed at approximately 200bp from the end of the insert.

Sequencing of the insert was carried out using a Sequenase Version 2.0® kit for the incorporation and termination reactions. A primer:template ratio of 1pmol:4µg was used, enabling approx. 200 to 300bp to be read per run on average. The sense-strand sequence data attained was used to synthesise 20-mer oligonucleotides primers that enabled the anti-sense strand to be sequenced.

## 7.4 λ6 encodes a multiple peripheral gene cluster.

The full sequence is presented in Figure 7.6, scrutiny revealed that there was not one β/α pair, as originally presumed on the fragment, rather three i.e., six genes in total, which have been designated (5' → 3') sxaBA, sxbBA and sxcBA. The gene sequence on the 2.1kb SalI fragment, however, at the 5' end was incomplete; there was only sequence to encode the last 50 amino acids from sxaA. The remaining sequence of the gene pair was determined by using the known sequence to design another oligonucleotide primer to walk upstream in the 5' direction. This was done by Dr R.C. MacKenzie, University of Glasgow. At the time of writing the upstream

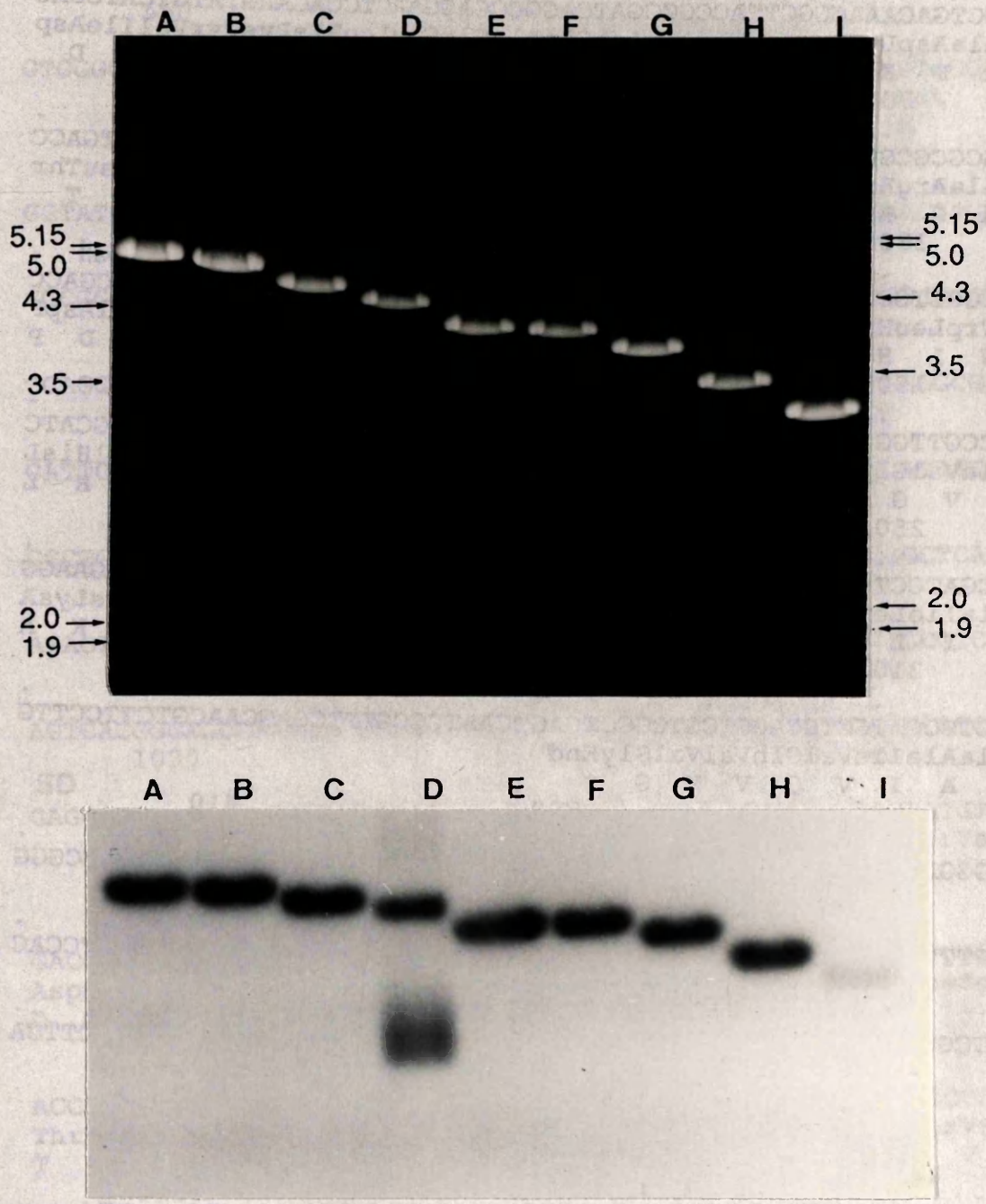



**Figure 7.5:** The set of nested plasmids (linearised with EcoRI), were probed with pLHIISB18. A signal could be detected until the final plasmid indicating that the β/α pair was approximately 200bp from the end of the insert.

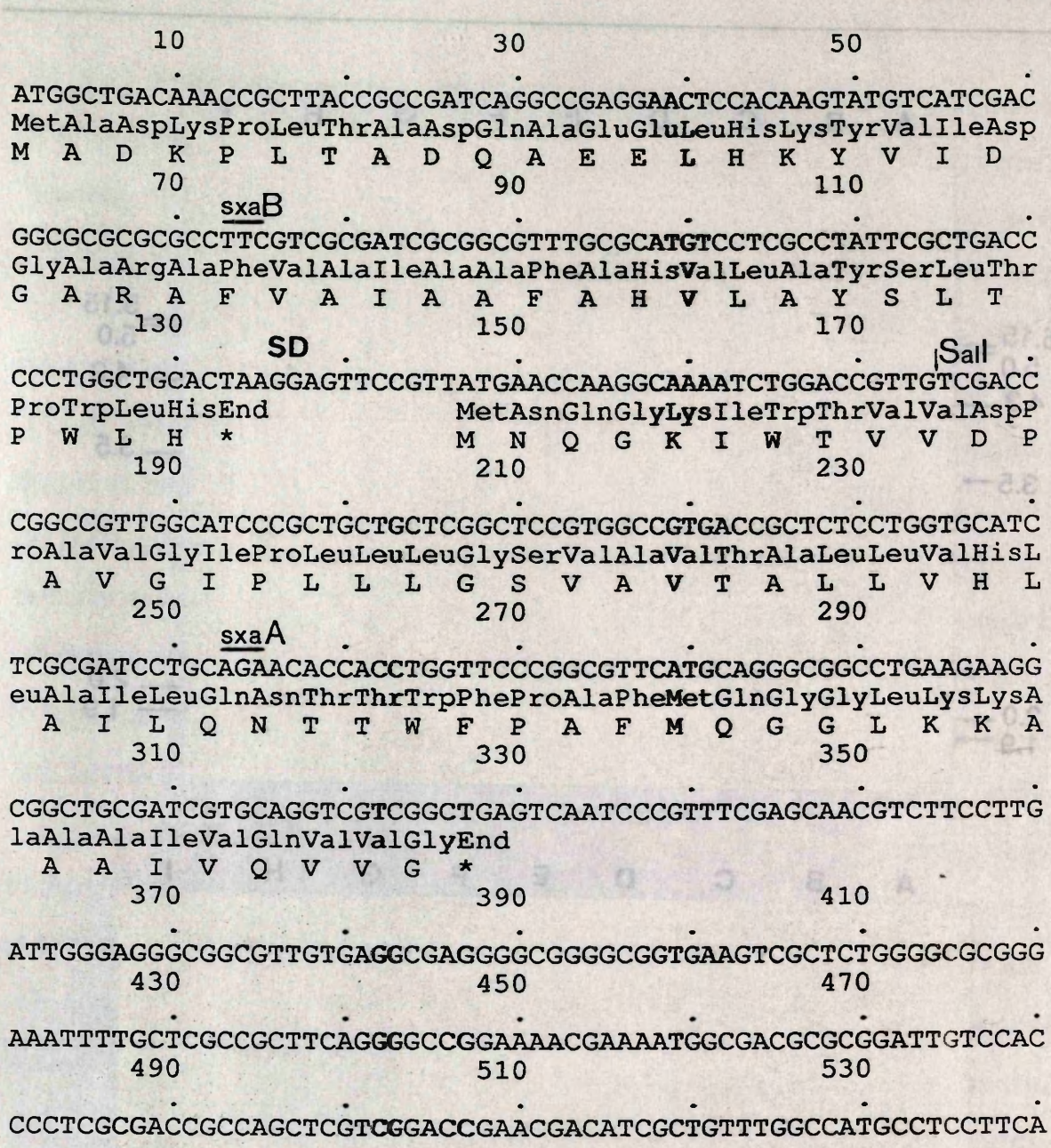
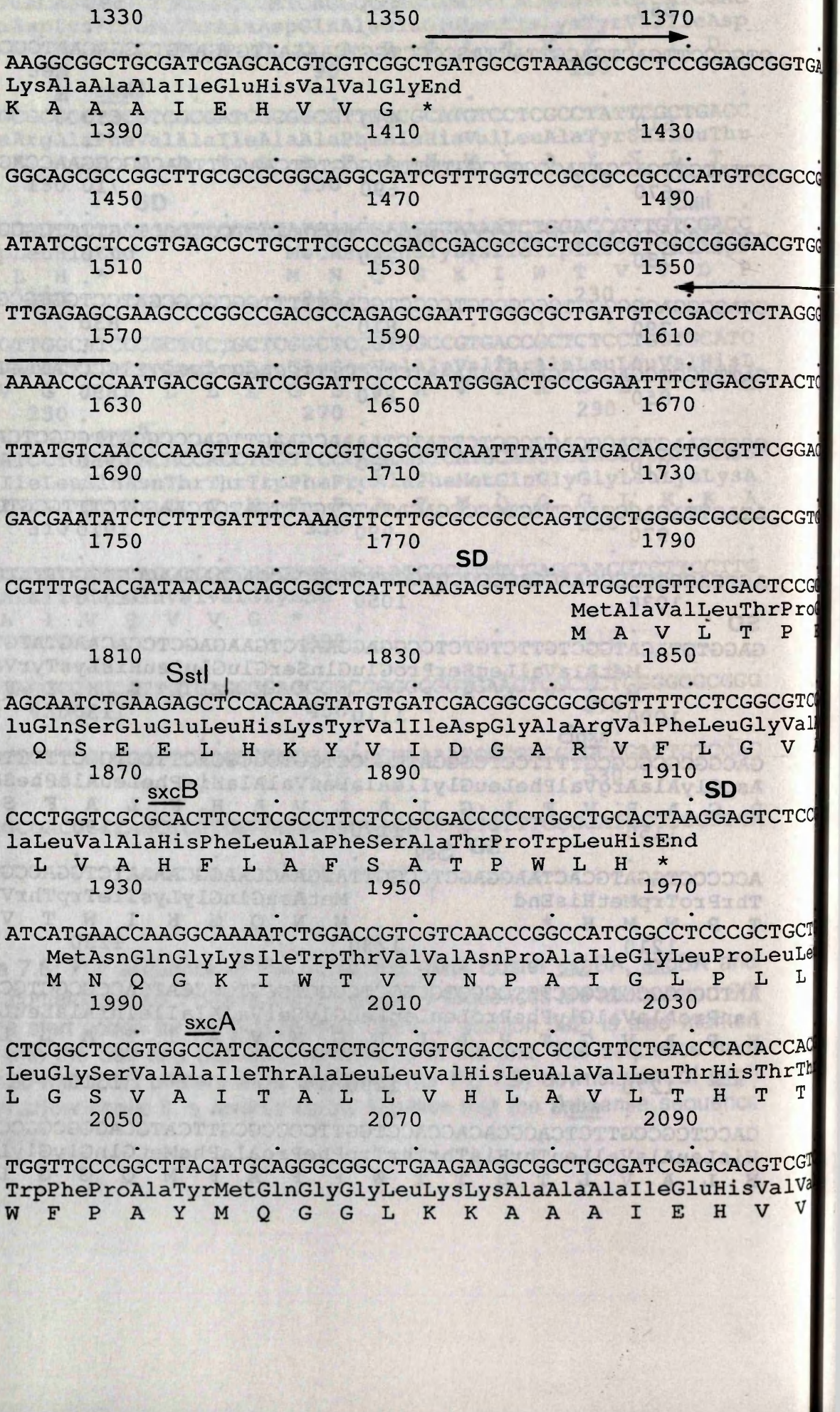



**Figure 7.6:** Full sequence of the treble β/α gene cluster sxaBA, sxbBA and sxcBA contained in λ6. Putative Shine-Dalgarno sequences are abbreviated SD and the start codon for the prospective ORF, at position 592, is also marked along with the SalI and SstI sites referred to in the text. The sequence from which 20-mer PCR primers were designed (for Fig 7.9) downstream of sxbA has an arrow above it, a reverse arrow signifies that the antisense sequence was used.

```
      550                   570                   590
      .                     .                     .
GTGGGCTTGACTGACGTTACTAGCCGCTGGTCAAGAATGTGAAGTCGGACAATGCCGGTC
                                                  Met
                                                  M
      610                   630                   650
      .                     .                     .
GGTATCTTGACGATACCGGCCGGTTTTTTGCGTGTCAAGTTTGACCGCCGAACCAGGCGG
      670                   690                   710
      .                     .                     .
CGCGGGGCGAGAACCTCAGCCCCCAGACCCTTCCCATCTCCCCCCTCCTTCTCGCGGCGC
      730                   750                   770
      .                     .                     .
CCAGCGGACCGGCGTCGCGCCGTCGCCTGCAGTTTTTGGCGCGCGATCCTGGGGCGGGGG
      790                   810                   830
      .                     .                     .
CATTCCCTGGAGGCGGAGGGGAAAATCTCCCTTTCCAGGTCTGGTTTCCCCATAGTATTC
      850                   870                   890
      .                     .                     .
GGCTGAACTCACCGACGCGCTCTTATGTAAAACGAAGTTGACCCGCCTTGGGCTCAATTT
      910                   930                   950
      .                     .                     .
ATACTTACACCTACGTTCTGGCCGAGATATCTGTTTGATCTCAAGGTCTTTGCGTCGCTC
      970                   990                   1010
      .                     .                     .
AGTCACGGGAGCGAAGGACCCGCGTGCGTTTGGCACGAGAAGAACCGTGGCTTATCATAA
      1030                  1050                  1070
SD    .                     .                     .
GAGGTGTACATGGCTGTTCTGTCTCCGGAGCAATCTGAAGAGCTCCACAAGTATGTGATC
        MetAlaValLeuSerProGluGlnSerGluGluLeuHisLysTyrValIle
        M  A  V  L  S  P  E  Q  S  E  E  L  H  K  Y  V  I
      1090                  1110                  1130
        .  sxbB             .                     .
GACGGCGCGCGCGTTTTCCTCGGCATCGCCCTGGTCGCGCACTTCCTCGCTTTCTCGATG
AspGlyAlaArgValPheLeuGlyIleAlaLeuValAlaHisPheLeuAlaPheSerMet
D  G  A  R  V  F  L  G  I  A  L  V  A  H  F  L  A  F  S  M
      1150                  1170                  1190
        .     SD SstI       .                     .
ACCCCCTGGATGCACTAAGGAGCTCCGGTTATGAACCAAGGCAAAATCTGGACCGTCGTC
ThrProTrpMetHisEnd          ↑  MetAsnGlnGlyLysIleTrpThrValVal
T  P  W  M  H  *               M  N  Q  G  K  I  W  T  V  V
      1210                  1230                  1250
      .                     .                     .
AATCCGGCCGTCGGCTTTCCGCTGCTGCTCGGCTCCGTGGCCATCACCGCGCTGCTGGTG
AsnProAlaValGlyPheProLeuLeuLeuGlySerValAlaIleThrAlaLeuLeuVal
N  P  A  V  G  F  P  L  L  L  G  S  V  A  I  T  A  L  L  V

      1270                  1290                  1310
        .  sxbA             .                     .
CACCTCGCCGTTCTCACGCACACCACCTGGTTCCCCGCGTTCATGCAGGGCGGCCTGAAG
HisLeuAlaValLeuThrHisThrThrTrpPheProAlaPheMetGlnGlyGlyLeuLys
H  L  A  V  L  T  H  T  T  W  F  P  A  F  M  Q  G  G  L  K
```


1330
1350
1370
AAGGCGGCTGCGATCGAGCACGTCGTCGGCTGATGGCGTAAAGCCGCTCCGGAGCGGTGA
LysAlaAlaAlaIleGluHisValValGlyEnd
K A A A I E H V V G *
1390
1410
1430
GGCAGCGCCGGCTTGCGCGCGGCAGGCGATCGTTTGGTCCGCCGCCGCCCATGTCCGCCG
1450
1470
1490
ATATCGCTCCGTGAGCGCTGCTTCGCCCGACCGACGCCGCTCCGCGTCGCCGGGACGTGG
1510
1530
1550
TTGAGAGCGAAGCCCGGCCGACGCCAGAGCGAATTGGGCGCTGATGTCCGACCTCTAGGG
1570
1590
1610
AAAACCCCAATGACGCGATCCGGATTCCCCAATGGGACTGCCGGAATTTCTGACGTACTC
1630
1650
1670
TTATGTCAACCCAAGTTGATCTCCGTCGGCGTCAATTTATGATGACACCTGCGTTCGGAC
1690
1710
1730
GACGAATATCTCTTTGATTTCAAAGTTCTTGCGCCGCCCAGTCGCTGGGGCGCCCGCGTC
1750
1770
1790
SD
CGTTTGCACGATAACAACAGCGGCTCATTCAAGAGGTGTACATGGCTGTTCTGACTCCGC
MetAlaValLeuThrPro
M A V L T P
1810
1830
1850
SstI
AGCAATCTGAAGAGCTCCACAAGTATGTGATCGACGGCGCGCGCGTTTTCCTCGGCGTC
luGlnSerGluGluLeuHisLysTyrValIleAspGlyAlaArgValPheLeuGlyVal
Q S E E L H K Y V I D G A R V F L G V
1870
1890
1910
sxcB
SD
CCCTGGTCGCGCACTTCCTCGCCTTCTCCGCGACCCCCTGGCTGCACTAAGGAGTCTCC
laLeuValAlaHisPheLeuAlaPheSerAlaThrProTrpLeuHisEnd
L V A H F L A F S A T P W L H *
1930
1950
1970
ATCATGAACCAAGGCAAAATCTGGACCGTCGTCAACCCGGCCATCGGCCTCCCGCTGCT
MetAsnGlnGlyLysIleTrpThrValValAsnProAlaIleGlyLeuProLeuLe
M N Q G K I W T V V N P A I G L P L L
1990
2010
2030
sxcA
CTCGGCTCCGTGGCCATCACCGCTCTGCTGGTGCACCTCGCCGTTCTGACCCACACCAC
LeuGlySerValAlaIleThrAlaLeuLeuValHisLeuAlaValLeuThrHisThrTh
L G S V A I T A L L V H L A V L T H T T
2050
2070
2090
TGGTTCCCGGCTTACATGCAGGGCGGCCTGAAGAAGGCGGCTGCGATCGAGCACGTCGT
TrpPheProAlaTyrMetGlnGlyGlyLeuLysLysAlaAlaAlaIleGluHisValVa
W F P A Y M Q G G L K K A A A I E H V V

```
      2110                2130                2150
        .                   .                   .                   .
GGCTGATTGCGTAAAGCCGCTCGGCAGGCTTGAGCGGCGGGACCGGGCCAAGGCCCGGCG
GlyEnd
G  *
      2170                2190                2210
        .                   .                   .                   .
CGACCAGCTCCGGATGCGGACAGTCCACCCCGCCTGGAGCCTGTCGAACGAAAACCAAGA
      2230                2250                2270
        .                   .                   .                   .
TGCGGCCGGCATTGTGACGATGCCGGGCGCTTTCTTTTGTCGTGTTCAGGCCGCCAGCAG
      2290
        .                   .
CTCCTCCAGCGCGGCGAGGTCGA
```

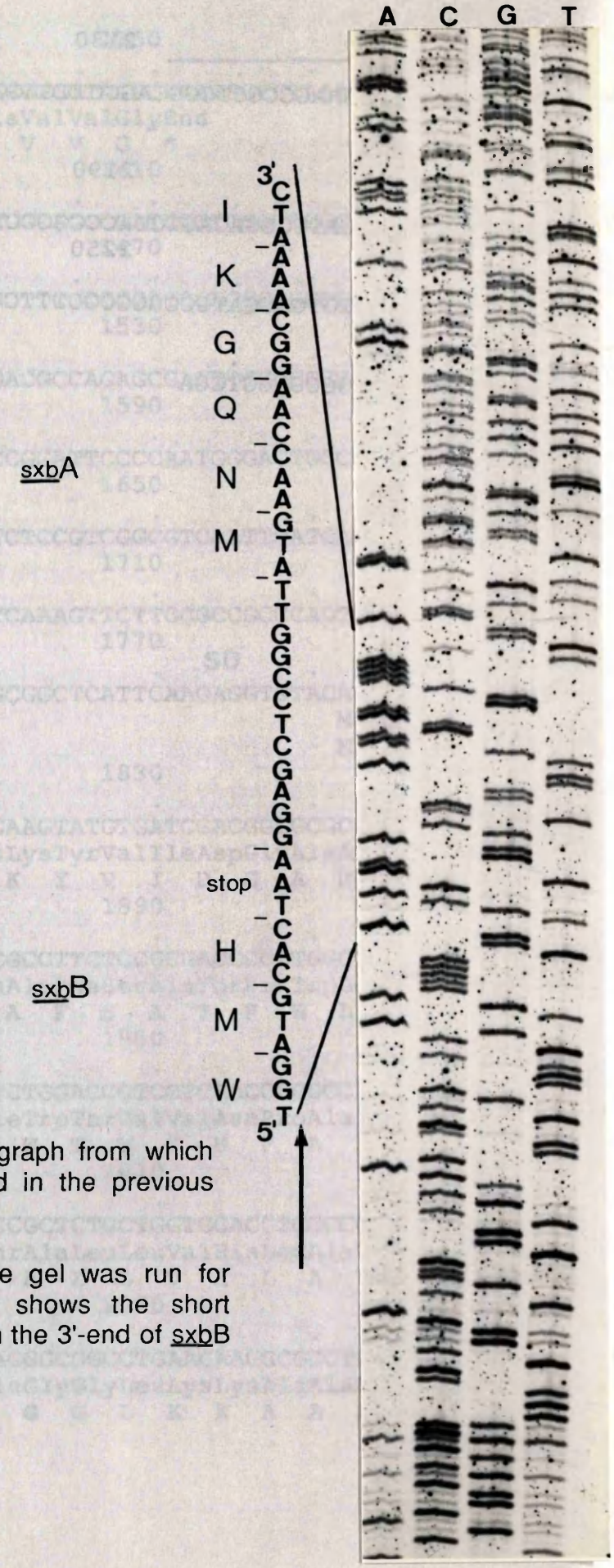



**Figure 7.7:**

Typical sequencing autoradiograph from which the sequence data presented in the previous figure was compiled.

This particular 6% acrylamide gel was run for approximately 3 hours and shows the short intervening sequence between the 3'-end of sxbB and the 5'-start of sxbA.

region of sxaB had not yet been determined. An example of a typical sequencing autoradiograph is illustrated in Figure 7.7.

The following features are worthy of comment; the two intervening regions (between sxaA & sxbB and sxbA & sxcB) are 700 and 429bp long and the former contains an open reading frame of 84 amino acids with the putative Met start codon at position 592. Upstream of sxbB and sxcB are short regions which can be tentatively identified as Shine-Dalgarno (SD) ribosome binding sites and immediately downstream of the β-gene of all three pairs is the identical sequence GGAG, which could act as another putative SD site, presumably to ensure the ribosome is retained on the message so that the α-gene is translated. The overall G+C content is 62% which is slightly less than reported previously for other members of the Rhodospirillaceae.

Complementary to the examination of the structural genes is the analysis of the non-coding regions, as it is these regions which are responsible for gene regulation and expression. Sequence data can be compared using 'Bestfit' analysis: this program searches for an optimal alignment of the best segment of similarity between two sequences and is available as part of a comprehensive software package from the 'Genetics Computer Group', University of Wisconsin.

The two intervening regions, after the stop codon at the C-terminal of the α-polypeptide from sxaA and prior to the start codon of sxbB (bases 329 → 1029 in Fig. 7.6), and the analogous region between sxbA and sxcB (bases 1352 → 1781) were compared using 'Bestfit' analysis. Figure 7.8 illustrates that there is 72% identity between the 232 bases proximal to these two gene pairs. This high degree of identity between two DNA sequences is usually indicative of a gene duplication event i.e., the addition of contiguous extra copies of a particular sequence through some recombination based mechanism within the cell. Thus, the high extent of homology present in the two intervening regions suggests that this treble gene cluster sxaBA, sxbBA and sxcBA, has arisen from one of the three gene pairs. Divergence will have occurred because the copies are able to undergo repeated

```
          Gap Weight:  5.000      Average Match:  1.000
       Length Weight:  0.300   Average Mismatch: -0.900

             Quality:   79.4             Length:    232
               Ratio:  0.354              Gaps:      5
Percent Similarity: 72.646   Percent Identity: 72.646

6bupstrm.Txt x 6cupstrm.Txt August 5, 1992  21:29  ..

                .         .         .         .         .
470 GGGAAAATCTCCCTTTCCAGGTCTGGTTTCCCCATAGTATTCGGCTGAAC 519
    ||||||| | |  |  |  | || || |||||||  |     | | |||
206 GGGAAAACCCCAATGACGCGATCCGGATTCCCCAATGGGACTGCCGGAAT 255
          .         .         .         .         .
520 TCACCGACGCGCTCTTATGTAAAACGAAGTTGACCCGCCTTGGGCTCAAT 569
    | | ||||  |||||||||| || | |||||||  |  | | ||  |||||
256 TT.CTGACGTACTCTTATGTCAACCCAAGTTGATCTCCGTCGGCGTCAAT 304
          .         .         .         .         .
570 TTATACTTACACCTACGTTCTGGCCGAG.ATATCTGTTTGATCTCAAGGT 618
    ||||  | |||||| ||||| | |  | |||||| |||||| |||| ||
305 TTATGATGACACCTGCGTTCGGACGACGAATATCTCTTTGATTTCAAAGT 354
          .         .         .         .         .
619 CTTTGCGTCGCTCAGTCACGGGAGCGAAGGACCCGCGTGCGTTTGGCACG 668
     ||||| ||| ||||| | || |||   ||||||||||||| |||||
355 TCTTGCGCCGCCCAGTCGCTGGGGCG.....CCCGCGTGCGTTT.GCACG 398
          .         .         .
669 AGAAGAACCGTGGCTTATCATAAGAGGTGTAC 700
    | || ||| | |||| ||   ||||||||||
399 ATAACAACAGCGGCTCAT.TCAAGAGGTGTAC 429
```

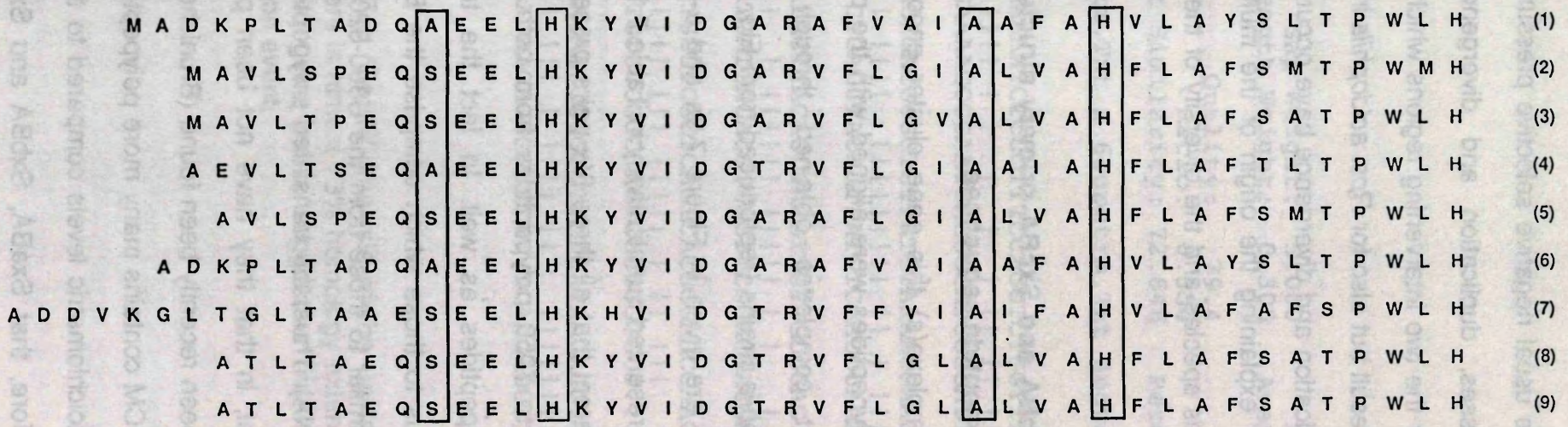
**Figure 7.8:** 'Bestfit' analysis of the upstream sequences of sxbB and sxcB. Thus there is almost 73% homology extending 232bp immediately upstream of these two gene pairs, suggestive that these have arisen through a gene duplication event.

mutation without being subjected to the usual negative selective pressure. These two fundamental evolutionary processes, duplication and divergence, have important consequences, not simply for the two intervening regions which may (or may not) have different functions as a result but also for *Rps. acidophila* in general. The knowledge from this data that duplication and divergence have occurred could provide eventually a rational basis for explaining the origin of the multiplicity of peripheral antenna genes present in this species and the diversity of the requisite control pathways.

## 7.5 Alignments of the SxaBA, SxbBA and SxcBA primary structures with published *Rps. acidophila* polypeptide sequences.

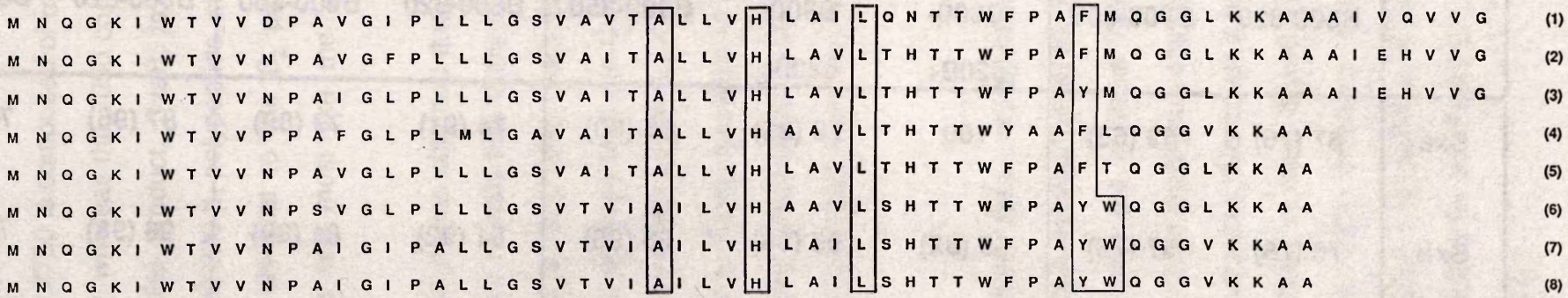
In order to establish which complexe(s) this gene cluster encodes, the primary sequence of the translated polypeptides were aligned with the published primary sequence data of different complexes obtained through Edman degradation for the various *Rps. acidophila* strains (reproduced from Fig. 1.7a and 1.7b). The alignments for the β-genes are given in Figure 7.9a and α-genes in 7.9b, furthermore, the homologies are presented quantitatively in Table 7.1.

Hence, from Table 7.1 it is apparent that all three β/α pairs present on λ6 have considerable similarity not only to 7050 polypeptides from both types of complex but also to the 7750 polypeptides as well. In fact the translated polypeptides are extremely homologous to those which comprise the B800-820 complex from 7750 and relatively dissimilar to those from the B800-820 of 7050 (the library was prepared from 7050 DNA). Thus, the translated polypeptides from this treble multigene cluster are novel, in that they have not been previously isolated or sequenced. Evidence has been recently been found (Brunisholz, R A., pers. comm.) that the *Rps. acidophila* ICM contains many more polypeptides than originally thought, albeit at very low stoichiometric levels compared to the 'main' complex present. It is possible therefore, that SxaBA, SxbBA and SxcBA are




(1) SxaB (3) SxcB (5) 7750 B800-820$\beta_2$ (7) 7050 B800-850$\beta$ (9) 10050 B800-850$\beta$
(2) SxbB (4) 7050 B800-820$\beta$ (6) 7750 B800-820$\beta_1$ (8) 7750 B800-850$\beta$

**Figure 7.9a:** Comparison of the published [from Zuber, 1990] *Rps. acidophila* primary sequences for the β-peripheral apoproteins with those obtained from λ6. Aligned on the conserved dimeric Bchl A-x-x-x-H binding motif and putative monomeric Bchl binding site.




(1) SxaA (3) SxcA (5) 7750 B800-820α (7) 7750 B800-850α
(2) SxbA (4) 7050 B800-820α (6) 7050 B800-850α (8) 10050 B800-850α

**Figure 7.9b:** Alignments of published [from Zuber, 1990] *Rps. acidophila* primary sequences for the α-peripheral apoproteins with those obtained from λ6. Aligned on conserved Bchl binding motif A-x-x-x-H-x-x-x-L. The aromatic residues at the C-terminal end postulated to play a role in determining the precise absorption maximum of the dimeric Bchl are also boxed.

| | BETA | | | | | ALPHA | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 7050 B800-820 | 7050 B800-850 | 7750 B800-820$ß_1$ | 7750 B800-820$ß_2$ | 7750 B800-850 | 7050 B800-820 | 7050 B800-850 | 7750 B800-820 | 7750 B800-850 |
| Sxa | 67 (76) | 59 (68) | 100 | 70 (85) | 68 (80) | 74 (91) | 77 (89) | 87 (96) | 79 (91) |
| Sxb | 70 (78) | 57 (73) | 65 (82) | 95 (98) | 83 (90) | 81 (92) | 81 (89) | 96 (98) | 77 (91) |
| Sxc | 73 (78) | 59 (73) | 67 (73) | 90 (93) | 90 (95) | 81 (94) | 83 (89) | 94 (98) | 81 (91) |

**Table 7.1:** 'Bestfit' analysis of identity (and similarity) of the apoproteins idenitfied from λ6 against all the respective polypeptides from the peripheral complexes of *Rps. acidophila* strains 7050 and 7750 that have been sequenced through Edman degradation. It is evident that SxaB apoprotein is identical to the $\beta_2$ apoprotein and that both SxbB and SxcB are most like the $\beta_1$ apoprotein from the 7750 B800-820 complex. Similarly, the respective α-apoproteins are most like the α-apoprotein from this complex.

responsible for some of these low level polypeptides and that the genes for the 'main' complex have not yet been cloned.

The B800-820 complex from 7750 is composed of α, $\beta_1$ and $\beta_2$ polypeptides (presumably in 2:1:1 stoichiometry), whereas the 7050 complex comprises only α- and β-polypeptides. The sxaB gene product, SxaB, is 100% identical to the published sequence for a 7750 B800-820 $\beta_1$ polypeptide, similarly SxbB and SxcB β-polypeptides are 95 and 90% identical to the $\beta_2$ polypeptide of the same complex. All three α-polypeptides are most like the α-polypeptide from this complex.

Comparison of the primary structures obtained through Edman degradation and DNA sequencing in Figure 7.9b, reveals that there is a significant degree of post-translational processing of the α-apoprotein. Both B800-820 and B800-850 have 8 and 7 residues respectively removed from the C-terminal end to yield the mature polypeptide. Furthermore, within the final 8 residues of the cleavage point and two after it, there is almost 100% identity between all the sequences, as well as a large degree of homology in the cleaved polypeptide itself. This is suggestive that a secondary structural motif forms that identifies the cleavage site to the protease.

## 7.6 This multigene cluster from *Rps. acidophila* strain 7050 has an analogous counterpart in *Rps. acidophila* strain 7750.

These results suggest that there are in strain 7050 a set of genes (i.e. sxaBA, sxbBA and sxcBA) which have an analogous copy in 7750 and when (or if) expressed give rise to a B800-820 complex. Moreover it has already been shown that there are in *Rps. acidophila* 7050 at least 8 and possibly as many as 14 different β/α gene pairs, (M$^{ac}$Kenzie, 1991) therefore, it appears that 3 of these gene pairs correspond to sxaBA, sxbBA and sxcBA, and that one of the remaining, as yet uncharacterised gene pairs codes for the B800-820 complex that has been sequenced through Edman degradation.

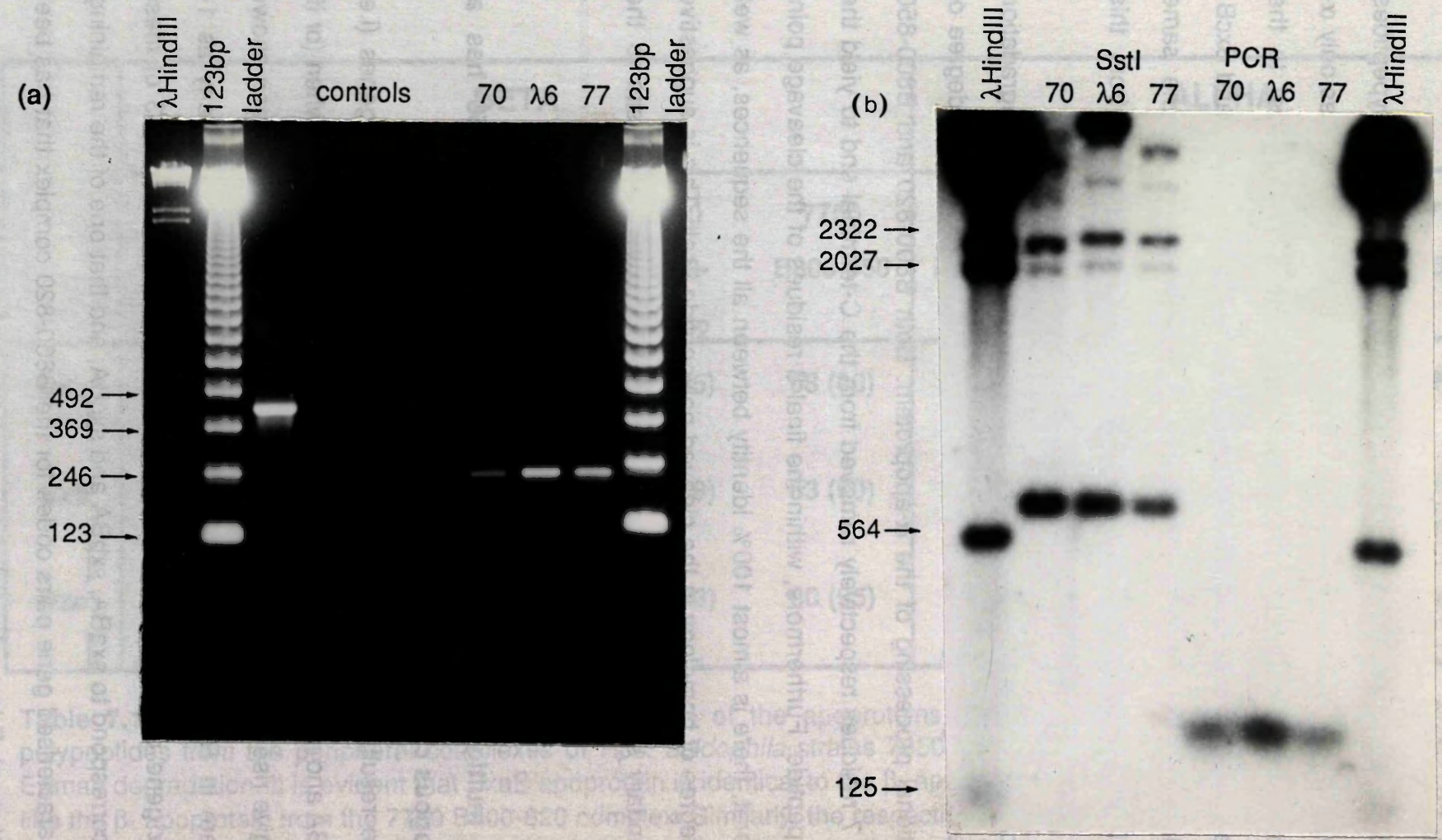



**Figure 7.10: (a)** Using sequence data from Figure 7.6 to design suitable primers, PCR was used to amplify 217bp immediately downstream of sixB. The PCR product is obseved when λ6 as well as 7050 DNA is the the template, however 7750 DNA also yields a similar sized fragment indicating the two strains are very homologous in this region. Furthermore, **(b)** sequence analysis revealed the amplified product is contained on a 652bp SstI fragment, which was identified when 7750 as well as 7050 genomic DNA were probed with the λ6 PCR product

To test the hypothesis that the DNA sequence in 7050, which contains sxaBA, sxbBA and sxcBA, has an analogous, extremely homologous counterpart in 7750 comprising the genes encoding the $\alpha$, $\beta_1$ and $\beta_2$ polypeptides, the following experiments were undertaken. Using the sequence presented in Fig 7.6, 20-mer primers were designed to amplify up the 217bp sequence immediately downstream of sxbA using polymerase chain reaction (PCR). The rationale for this was as follows; this sequence precedes the region identified as having arisen through gene duplication, therefore, can be assumed to be related to a putative archetypal 'acidophila'-like organism and not involved in the regulation of these genes. As such it should not be subject to the same degree of constraint, and divergence between 7050 and 7750 will be greatest. In fact Figure 7.10a illustrates that the primers were able to amplify an identically sized fragment from genomic 7750 DNA as well as from genomic 7050 and λ6 DNA, strongly suggestive that this region is very highly conserved in both strains. This conclusion was reinforced when λ6 PCR product was radiolabelled and used to probe a Southern Blot of genomic 7750 and 7050 DNA cut with SstI. From Figure 7.6, sites for this enzyme are present at positions 1164 and 1816 and so the λ6 PCR product sequence is contained within a 752bp fragment. Probing should detect a band of this size in genomic 7050 and λ 6 DNA; if the SstI sites have been conserved a similar sized fragment will also be observed for genomic 7750 DNA; that this is in fact the case is shown in Figure 7.10b and provides powerful evidence for the above hypothesis.

## 7.7 **Conclusions.**

This chapter has shown that an EMBL3 clone, λ6, derived from *Rps. acidophila* 7050 DNA contains three B800-820 β/α gene pairs which appear to have arisen through gene duplication events; none of the three gene pairs when translated match the published primary sequence for 7050 B800-820. Indeed primary sequence analysis revealed that they are very closely related to the published data for the polypeptides present in the B800-820 complex from strain

7750. Experiments were performed which showed that the DNA sequence that gives rise to sxaBA, sxbBA and sxcBA, has an extremely homologous counterpart in 7750. The logical deduction is that gene duplication events occurred to give rise to this multigene family before the divergence of the two strains into *Rps. acidophila* 7050 and 7750, and that divergence within each multigene family has been severely constrained due to the selective advantage that expression of this complex confers.

# Chapter Eight

## Mobilisation of *Rps. acidophila* strain 7050 peripheral antenna complex genes into *Rhodospirillum rubrum* strain S1

## 8.1 Introduction.

This chapter was intended to determine, primarily if the method developed in Chapter 6 for introducing recombinant DNA, such as that identified in Chapter 7, was feasible in practise. Secondly to assess if the genes borne on the 2.1kb SalI fragment which was cloned and sequenced in the previous chapter, could be constitutively induced and thereby produce intact, functional ICM-bound light-harvesting complexes. This fragment contains only the 3'-end of sxaA, in addition to complete copies of sxbBA and sxcBA. Therefore if the promoter regions for these genes are situated upstream of sxaB, they are not present and meant that, in effect, if transcription was to be possible, in the context of this experiment, the necessary sequences for expression must lie in the intergenic regions upstream of sxbBA and/or sxcBA.

## 8.2 Cloning and mobilisation of antenna genes.

As outlined previously (6.3.4) the 10.6kb $Tc^r$, $Mob^+$ plasmid pRK404 (Ditta *et al.,* 1985) was able to be introduced into the Rhodospirillaceae with a transfer efficiency of $10^{-3}$-$10^{-4}$. During the elapsed time between completion of these experiments and those now proposed an improved construct became available: pRK415 (Keen *et al.,* 1988). This plasmid differs from its predecessor in that it contains the polylinker from pUC19 rather than pUC9 and the EcoRI site outside the polylinker was deleted: these changes have no effect upon the mobilisation properties of the plasmid. The restriction map of this plasmid is shown in Figure 8.1a.

To excise the antenna complex genes plasmid pB52 was digested with the REs KpnI and HindIII, the 2.1kb fragment separated from the vector by gel electrophoresis and purified away from contaminating agarose by GeneClean®. This fragment was directionally ligated into the KpnI/HindIII polylinker of pRK415 and the resulting construct named pRKB52. Finally both plasmids, pRKB52, (and pRK415 to act as a negative control), were transformed into the mobilisable *E. coli*

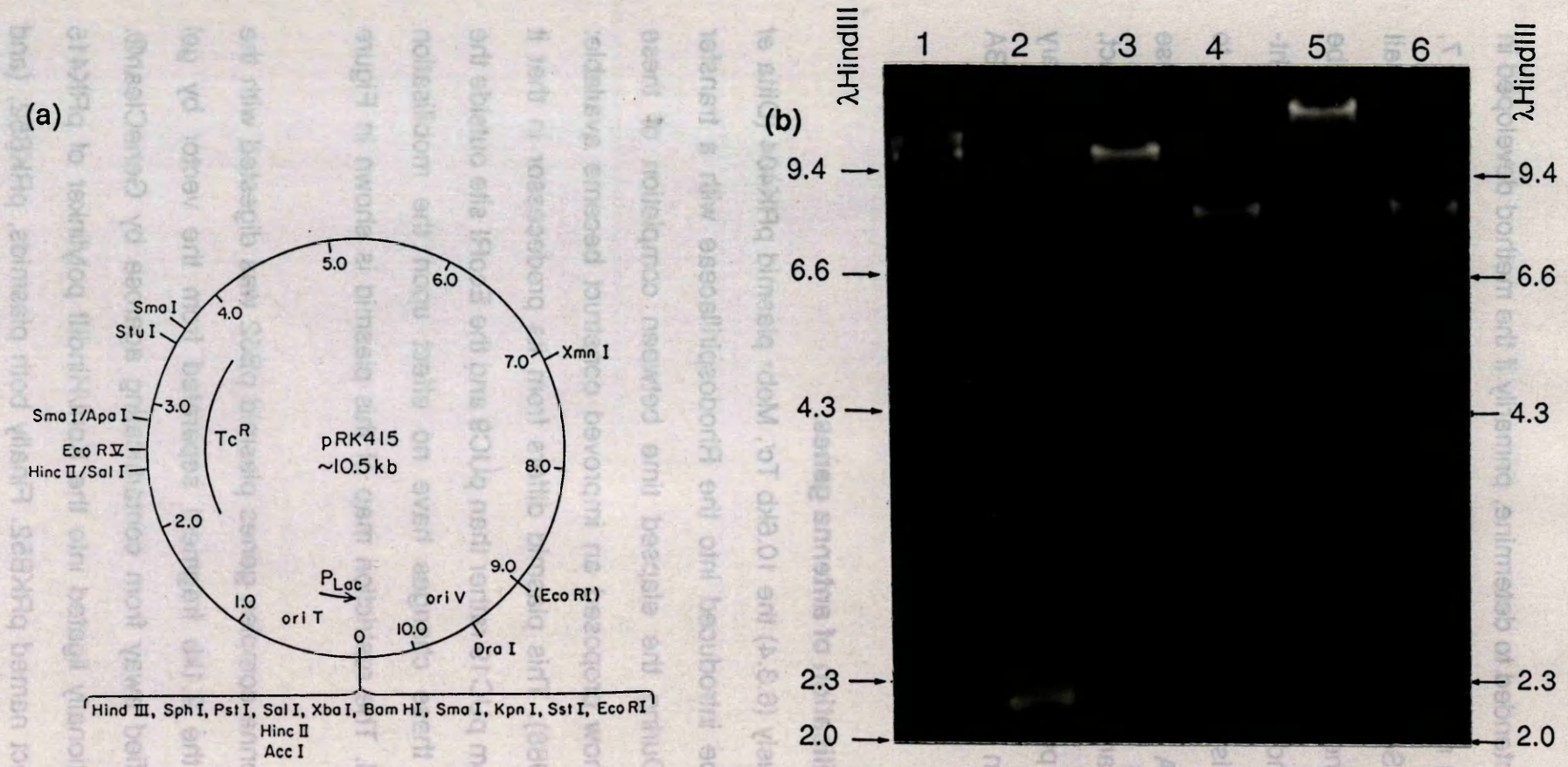



**Figure 8.1: (a)** Restriction map of pRK415 **(b)** Agarose minigel illustrating the cloning of the approx. 2.1kb SalI fragment that contains the antenna gene pairs into the mobilisable vector pRK415 to form pRKB52. Lane 1: pRK415 linearised with HindIII and KpnI, Lane 2: GeneClean® purified 2.1kb fragment excised from pB52 by HindIII and KpnI. These two DNA species were ligated together and transformed into *E. coli* Sm10, a Tc$^r$ colony was minipreped and cut with EcoRI (lane 5) and SalI (lane 6). Lanes 3 and 4 are the minipreped plasmid from a colony transformed with pRK415 i.e., as a control, cut with EcoRI and SalI respectively. As expected the DNA in lane 5 is greater than that in lane 1 by 2.1kb and the plasmid in lane 6 contains the extra SalI fragment containing the antenna complexes when compared with lane 4.

strain Sm10 with an efficiency of $1.5x10^4$ transformants/μg DNA. This process is illustrated in Figure 8.1b.

The next step involved conjugal mating to introduce these plasmids into a suitable Rhodospirillaceae recipient which has a negative peripheral antenna background i.e. B800-850$^-$. The most desirable species in view of the biochemical characterisation already carried out would have been *Rps. acidophila*; however, because of the existence of multiple peripheral genes combined with limited remaining time, it was thought that the production of a mutant incapable of producing peripheral antennae was not feasible. Another possibility was *Rb. sphaeroides* strain M21 which has a B800-850$^-$ phenotype produced through NG mutagenesis; this is an active line of research being pursued through collaboration with Dr. C. N. Hunter, University of Sheffield. The third possibility, *R. rubrum,* was chosen because this species contains only the B890 complex for its light harvesting capability, i.e. it is naturally B800-850$^-$.

A chemoheterotrophic *R. rubrum* S1 culture was grown from a single photoheterotrophic colony and mated with *E. coli* strain Sm10 harbouring either pRK415 or pRKB52. The resulting transfer efficiencies are presented in Table 8.1. Figure 8.2a illustrates the two different *R. rubrum* S1 recipients produced after mating colony regeneration and Figure 8.2b when grown in liquid culture.

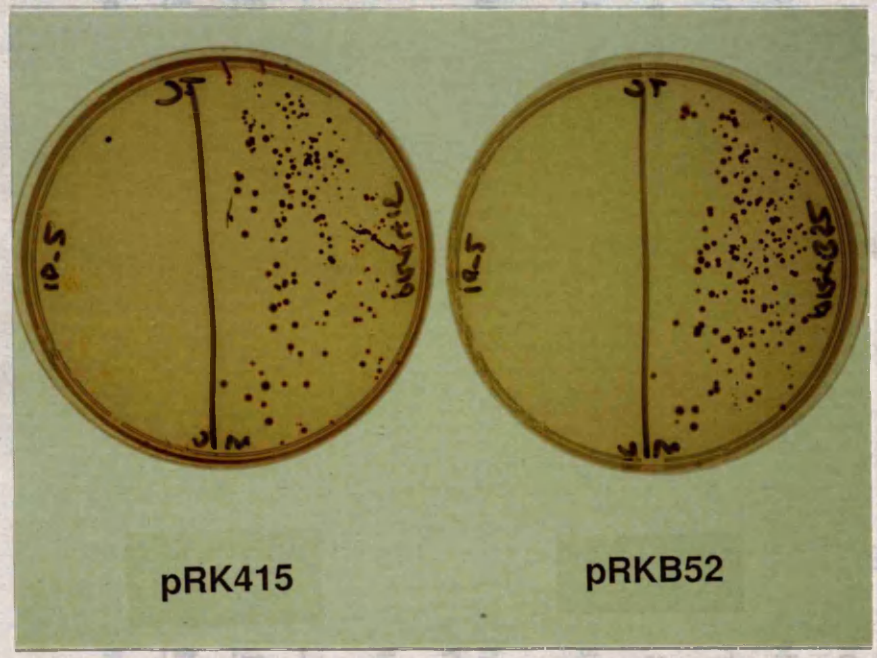
**Table 8.1: Mobilisation of *Rps. acidophila* 7050 antenna complex genes into *R rubrum* S1**

***E. coli* Sm10 x *R. rubrum* S1.**

| Plasmid Introduced | pRK415 | pRKB52 | pRK404 |
|---|---|---|---|
| Transfer Efficiency | $1.2x10^{-3}$ | $9.9x10^{-4}$ | $1.6x10^{-3}$ |

**The transfer efficiency with plasmid pRK404 is shown as a comparison**

(a)




(b)


**Figure 8.2:(a)** Plates after mating either *E. coli* SM10 [RK415] or [RKB52] with *R. rubrum* S1. The left side of each plate was plated with unmated *R. rubrum* S1 cells therefore all the cells are killed. **(b)** One colony from each plate was grown up in liquid culture and it is apparent that S1[RKB52] cells have an altered carotenoid composition.

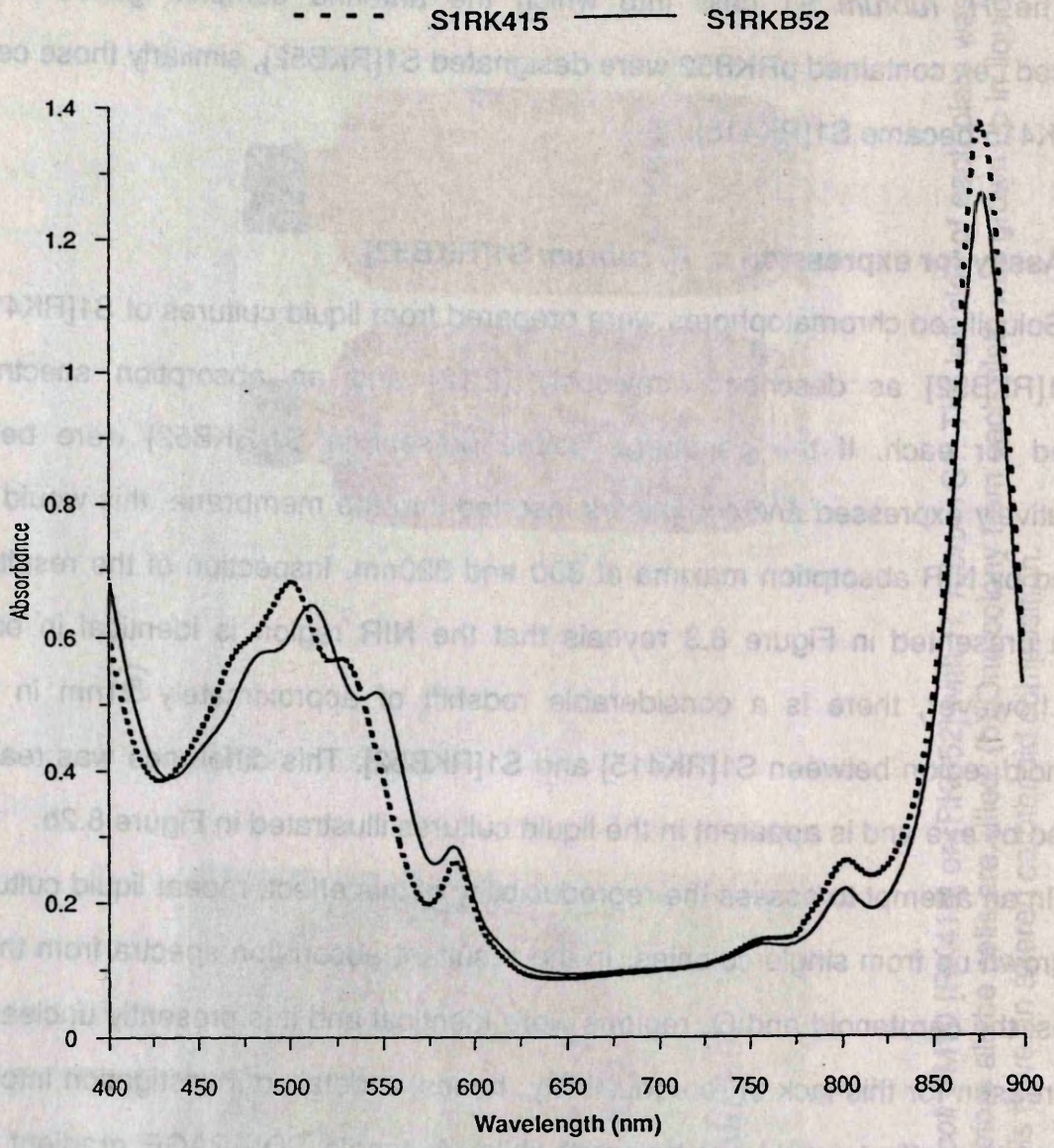
The *R. rubrum* S1 cells into which the antenna complex genes were introduced i.e., contained pRKB52 were designated S1[RKB52], similarly those cells with pRK415 became S1[RK415].

## 8.3 Assay for expression in *R. rubrum* S1[RKB52].

Solubilised chromatophores were prepared from liquid cultures of S1[RK415] and S1[RKB52] as described previously (2.12) and an absorption spectrum recorded for each. If the peripheral genes present in S1[RKB52] were being constitutively expressed and complexes inserted into the membrane, this would be detected by NIR absorption maxima at 800 and 820nm. Inspection of the resultant spectra presented in Figure 8.3 reveals that the NIR region is identical in each strain, however, there is a considerable redshift of approximately 11nm in the carotenoid region between S1[RK415] and S1[RKB52]. This difference was readily detected by eye and is apparent in the liquid cultures illustrated in Figure 8.2b.

In an attempt to assess the reproducibility of this effect, repeat liquid cultures were grown up from single colonies. In the resultant absorption spectra from these cultures, the carotenoid and $Q_y$ regions were identical and it is presently unclear as to the reason for this lack of reproducibility, hence, a detailed investigation into the cause of this effect would not be worthwhile. A simple SDS-PAGE gradient gel, however, would reveal any differences in the polypeptide composition that might be responsible for the difference in absorption in the respective chromatophores. This technique would also show if there had been any insertion of SxbBA and/or SxcBA polypeptides into the *R. rubrum* ICM. The resultant silver-stained gel is illustrated in Figure 8.4.

An absorption profile in the $Q_y$ region similar to that recorded in Fig. 8.3, would result if the polypeptides had been inserted into the ICM but did not bind Bchl. It is apparent, however, from Figure 8.4. that neither the low molecular weight SxbBA nor SxcBA polypeptides have been detected (lane 3), as judged against the homologous $\alpha$, $\beta_1$ or $\beta_2$ polypeptides from the *Rps. acidophila* 7750 B800-820




**Absorption Peaks in the Carotenoid Region (nm)**

| S1RK415 | S1RKB52 |
|---|---|
| 470.0 | 478.5 |
| 501.4 | 512.3 |
| 530.4 | 546.1 |

**Figure 8.3:** Chromatophores prepared from each culture in Fig. 8.2b were solubilised and absorption spectra recorded. It is apparent that S1[RKB52] has an identical absobance profile in the $Q_y$ region to S1[RK415] but that there is a redshift in the carotenoid region.

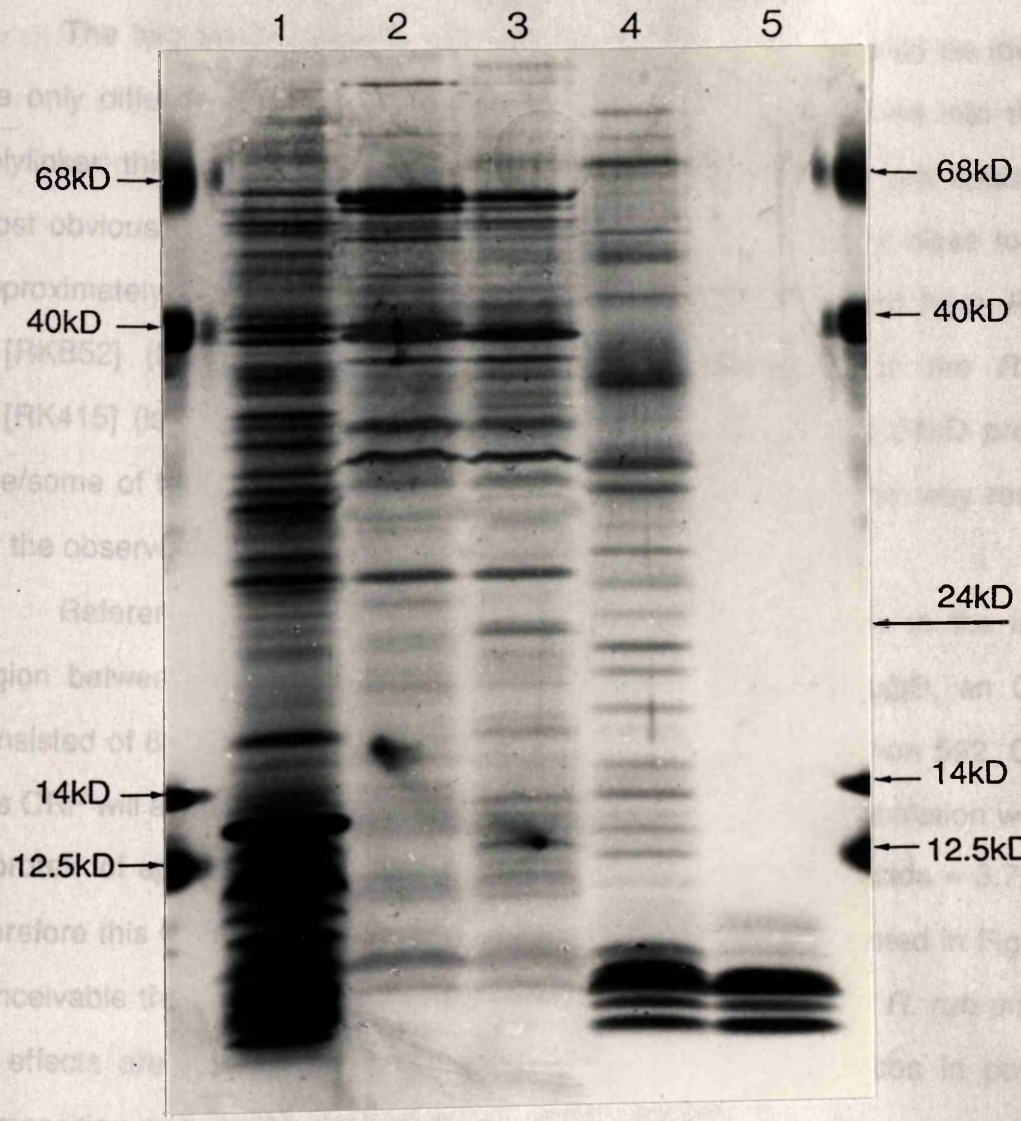



**Figure 8.4:** SDS-PAGE gradient (10-20%) gel demonstrating that there had been no insertion of either SxbBA or SxcBA polypeptides into the ICM. The left side arrows highlight (top) the most obvious difference between lanes 2 and 3 which is the appearance of an approx. 24kD polypeptide, and (bottom) the area where antenna polypeptides migrate.

Lane 1: *R. rubrum* S1 chromatophores.
Lane 2: *R. rubrum* S1[RK415] solubilised chromatophores.
Lane 3: *R. rubrum* S1[RKB52] solubilised chromatophores.
Lane 4: *Rps. acidophila* strain 7750 solubilised chromatophores.
Lane 5: *Rps. acidophila* strain 7750 B800-820 isolated complexes.

complex that are present at approximately 6kD (lanes 4 and 5). Thus the conclusion is reached that there has been no expression of these genes.

The two lanes containing the solubilised complexes should be identical as the only difference between them is the 2.1kb SalI insert cloned into the vector polylinker: this is plainly not the case as numerous differences are apparent. The most obvious are the two polypeptides that have migrated very close together at approximately 24kD in the solubilised chromatophores prepared from *R. rubrum* S1[RKB52] (lane 3). Similar polypeptides are not present in the *R. rubrum* S1[RK415] (lane 2) sample. The inference is made that these 24kD proteins (or one/some of the other less pronounced differences) are in some way responsible for the observed shift in the region between 400 and 550nm.

Reference to 7.3 and Fig. 7.6 will reveal that there was in the intergenic region between the 3'-end of sxaA and the start codon of sxbB, an ORF that consisted of 84 amino acids with its putative start codon at position 592. Obviously this ORF will also have been introduced into the recipient and translation would give a protein of approximately 9.3kD (assuming 1kb ≈ 333 amino acids ≈ $3.7 \times 10^{-4}$kD), therefore this ORF cannot be either of the 24kD proteins highlighted in Fig 8.4. It is conceivable though, that this gene is constitutively expressed in *R. rubrum* S1 and its effects are pleiotrophic, which would result in the differences in polypeptide composition and carotenoid absorption. No further experiments were performed to characterise the precise nature of these effects, therefore, their cause remains unknown.

## 8.4 **Conclusions.**

No evidence of the constitutive expression of sxbBA and sxcBA in *R. rubrum* S1 was obtained. However, the introduction of this fragment did appear to have some pleiotrophic effect on the carotenoid composition of the cells, although this effect could not be shown to be reproducible.

It is apparent that in this particular case there were too many parameters that were ill-defined, caused primarily by the forced use of *R. rubrum* as the recipient,

and the lack of remaining time. In spite of the lack of expression and the reproducibility problem, the results presented in this chapter were deemed successful because they demonstrated that the method used to introduce recombinant DNA into recipient species/strains was feasible and consequently could in future experiments produce worthwhile results.

# Chapter Nine

# Discussion

## 9.1 Adaptation to non-optimal conditions.

Adaptation by members of the Rhodospirillaceae to non-optimal growth conditions, particularly decreasing light intensity, is already well characterised in the literature. The cells are able to increase the numbers of RC in the ICM and the size, whenever possible, of the PSU; which in conjunction with an increase in the overall amount of ICM per cell leads to a considerably enhanced light harvesting potential: important if the cells are to compete successfully for the available light.

Prior to this study two wild type isolates of *Rhodopseudomonas acidophila* had been reported to show a further type of adaptation to non-optimal growth conditions, namely the production of different types of peripheral antenna. Specifically strain 7050 produced Type II B800-850 and B800-820 in response to decreased light intensity (Cogdell *et al.,* 1983) whereas strain 7750 produced the same complexes in response to decreasing temperature (Zuber and Brunisholz, 1991). A third isolate, strain 10050, was available but its responses were as yet uncharacterised.

It was apparent that these three isolates when subjected to non-optimal growth regimes have the potential to provide an ideal, indeed unique, system in which to study photosynthetic gene organisation and their co-ordinate regulation. Furthermore, it was equally apparent that this study could only be undertaken through the application of molecular biological techniques. As a prerequisite, therefore, it was necessary to define the changes that occurred within each wild type strain in response to a progressive decrease in one parameter that effected non-optimal conditions. The results obtained would then act as a benchmark, a wild type reference, facilitating comparisons not only between strains but also the correlation of phenotypic mutants with their genotypes, enabling an overall understanding of the processes involved to be gained.

Two main types of growth regimes were used in this work to induce changes in the peripheral antenna; the light intensity available for photosynthesis was decreased (from approx. 160 to 4μmols/s/$m^2$ at 30$^{o}$C) and the temperature that the

cells were cultured was also lowered (from 30 to 20°C at 'high' [approx. 160µmols/s/m²] or 'low' [approx. 50µmols/s/m²] light intensities). It has to be borne in mind that these growth regimes have been designed within an experimental context; as such they are artificial in relation to a *Rps. acidophila* cell *in situ.* That is to say, a *Rps. acidophila* cell in a peat bog rarely experiences 160µmols/s/m² light at 30°C, moreover the light intensity and temperature parameters are not in reality independent of each other, rather they act in combination to ensure the light-harvesting capacity of the cell is optimum for its particular individual requirements.

### 9.1.1 *Rhodopseudomonas acidophila* strain 7750.

This strain was found to be able to produce B800-820 in place of B800-850 when grown at low light and decreased temperature. Furthermore, if the growth temperature was non-optimal but the incident light intensity was sufficiently great then the insertion of B800-820 would be prevented. This led to the conclusion that this strain has a light 'switch' in addition to a temperature 'switch' and that the regulatory mechanisms for the former take precedence i.e., they are able to overrule those of the latter. Indeed the situation is more complicated than this, because at extremely low light intensity (<4µmols/s/m²) or extremely low temperatures (<20°C) evidence was presented (Fig. 3.1, spectrum recorded at 4µmols/s/m²) that this strain is able to reinsert B800-850 into the ICM. This particular complex was not investigated further in this study, therefore, questions such as; is this the same complex as the high-light B800-850 complex, or what is its precise light-harvesting role, remain unanswered.

The finding that strain 7750 has a light intensity component to its regulation of peripheral antenna genes which is dominant over the temperature component, has important implications for the work presently underway to determine the structure of the B800-820 complex through X-ray crystallography (Guthrie *et al.*, 1992). Previously cells with their peripheral antenna consisting of B800-820 were harvested from 10l cultures that had been grown at 24°C. In view of these findings it seems probable that insertion of this complex was due primarily to low light (in such

a large culture flask the penetrating light would not be uniform: only relatively few cells on the outside would receive adequate light) with the temperature effect secondary.

The changes in the total carotenoid content of the cells in response to decreasing light intensity was also measured. These experiments revealed that the only change detected from HPLC analysis was an increase in the amount of unglucosylated rhodopin at low light cells when compared with high light grown cells. As the attachment of a sugar residue to the carotenoid has no effect on the light harvesting ability of the cell it seems probable that by decreasing the amount of gluconeogenesis this is an conservation strategy by the cell.

### 9.1.2 *Rhodopseudomonas acidophila* strain 7050.

In response to growth at progressively decreasing light intensities this strain was able to replace B800-850 with B800-820. Carotenoid analysis of whole cells revealed the main carotenoid present changed from orange-brown rhodopin glucoside to purple rhodopinal glucoside. This is presumably a physiological response related to the findings of Angerhofer *et al.,* (1983) who reported that the carotenoid-bacteriochlorophyll energy transfer efficiency for the isolated B800-820 complex was 70-75%, against 50-55% for the B800-850 complex. If these values are representative of the situation *in vivo* then rhodopinal containing cells will possess an advantage when subjected to growth at low light. Interestingly it became evident that these two low light adaptations are not induced simultaneously: rather the switch from rhodopin glucoside to rhodopinal glucoside begins before the insertion of B800-820 into the ICM in place of B800-850. Thus it is possible to culture *Rps. acidophila* strain 7050 cells that contain purple rhodopinal glucoside as well as B800-850. Nothing is as yet known regarding the regulatory pathways that control these effects, for example, if they are controlled by independent mechanisms, or if the mechanism is the same for both but the carotenoid component is more sensitive. In any event it is interesting to speculate that the reason that this effect has evolved is to confer on 7050 cells a degree of flexibility in

their response to decreasing light that would not be possible if the carotenoid switch and complex switch were simultaneous. This is to say that if the decrease in the available light is only moderate, then it would be metabolically expensive to synthesise a completely different type of complex to compensate, however, this could be accomplished by the production of rhodopinal from rhodopin requiring only a simple methyl to aldehyde group oxidation.

No evidence was obtained that this strain was able to alter either its peripheral antenna complement or its carotenoid composition in response to decreasing temperature, although evidence was presented that the carbon source on which the cells are cultured is able to influence peripheral antenna complement. A systematic study of this effect, first reported by Heinemeyer and Schmidt, (1983), could yield valuable information regarding the control of this type of complex and its relation (if any) to the redox potential of the cell.

### 9.1.3 *Rhodopseudomonas acidophila* strain 10050.

No evidence was obtained that this strain was able to show any adaptation, regarding either its type of peripheral antenna complex or its carotenoid composition, to growth at decreasing light intensity, temperature or carbon source. It has been shown that this strain possesses B800-820 genes (Barrett S. J. pers. comm.), therefore, the question that arises is; 'are expression of these genes possible ?' and if so what are the conditions necessary to achieve this. As none of the regimes above were able to induce expression it is not readily obvious what other growth regimes could succeed when these have failed. However, this dilemma could to an extent be circumvented in future work by exploiting conventional molecular biological techniques and those developed in Chapter 6 and 8 of this work. A simple example can easily be envisaged where B800-820 genes are placed downstream of (a) a 7050 B800-820 promoter sequence and (b) a similar putative sequence from 10050, in a suitable mobilisable vector. This could then be introduced into a 10050 mutant with a negative peripheral antenna phenotype and the cells grown at low light. If mutation(s) in the 10050 promoter

regions are responsible for non-expression at low light, then (a) will allow expression of the genes and B800-820 will be inserted into the ICM, whereas (b) will not. Hence a direct comparison of the two sequences would reveal the mutation(s) responsible.

Previous reports regarding *Rps. acidophila* peripheral antennae have referred to Type I B800-850 complex (Fig 1.5a) and a Type II B800-850 complex (Fig 1.5b) that was obligatorily associated with the B800-820 complex (Cogdell, 1986). This nomenclature arose because these studies always used cells that had been cultured at fixed light intensities i.e., one high light (Type I) and the other low light (Type II), however, the advantage of this study was that cells were cultured at progressively decreasing light intensities. This had the effect that the insertion and replacement of complexes could be understood in the context of the whole series. Comparison of Fig. 1.5b with the spectrum recorded at 40μmols/s/m$^2$ in Fig. 3.9 shows they are very similar, however, examination across the whole series reveals the continuity of the process in which the turnover of the peripheral antenna occurs according to the light intensity. It is evident that the replacement i.e., the diluting out, of the high light B800-850 (Type I) complex is balanced with the concomitant insertion of the B800-820 complex, and that the spectra previously thought to contain Type II B800-850 in conjunction with B800-820, is in fact merely the intermediate stage when the peripheral antenna is composed of approximately stoichiometric amounts of Type I B800-850 and B800-820. This being so then the use of Type I and II nomenclature is no longer relevant.

The previous study on *Rps. acidophila* 7050 molecular biology (M$^{ac}$Kenzie, 1991) revealed the large number of β/α gene pairs, possibly as many as 14, present on the 7050 genome. As the minimal number required is 2, i.e., one for B800-850 and the other B800-820, the remainder might be accounted for by three possibilities.

(1) The recent discovery by Brunisholz (pers. comm.) that the *Rps. acidophila* ICM contains many more peripheral antenna polypeptides than originally thought,

albeit at low levels relative to the main complex polypeptides. This provides evidence that at least some of these extra genes are expressed and raises the interesting question as to the role of these low level polypeptides, which presumably form functional complexes, in relation to the light-harvesting capability of the ICM.

(2) The insertion of B800-820 complex in *Rps. acidophila* 7750 has been shown to be dependent on light intensity and temperature. This complex could be transcribed off different gene copies, each of which was induced by only by one of these stimuli, (although if this was the case the light regulatory circuit must be able to repress the temperature induction). This accounts for only four gene pairs i.e., light induced $\alpha$, $\beta_1$, $\alpha$, $\beta_2$ and a temperature induced $\alpha$, $\beta_1$, $\alpha$, $\beta_2$. The previously mentioned finding that there appears to be reinsertion of the B800-850 complex at very low light and temperatures, would account for another two gene pairs if they are different from the high light B800-850 gene pair.

(3) Another possibility is that the expression of one or more of these other peripheral gene copies is induced under non-optimal growth conditions for which the external stimuli, because the cells are cultured in a laboratory environment, has not yet been discovered.

Conversely, many of these gene pairs may in fact be pseudogenes, i.e., non-expressible under any circumstances. If this is the case the obvious question is to why the cell has retained this non-functional DNA its genome, thereby incurring a continual heavy burden on the cell during replication and bearing in mind that bacterial recombination systems are extremely efficient in deleting excess DNA.

## 9.2 Cloning of *Rps. acidophila* peripheral antenna genes.

The cloning and sequencing of the multiple gene cluster sxaBA, sxbBA and sxcBA from 7050 in this work has revealed some interesting insights into some of the points raised above. The discovery that sxcBA has arisen from sxbBA (or vice versa) through a gene duplication event is direct evidence that other copies of peripheral antenna gene pairs may have also arisen by this mechanism. A

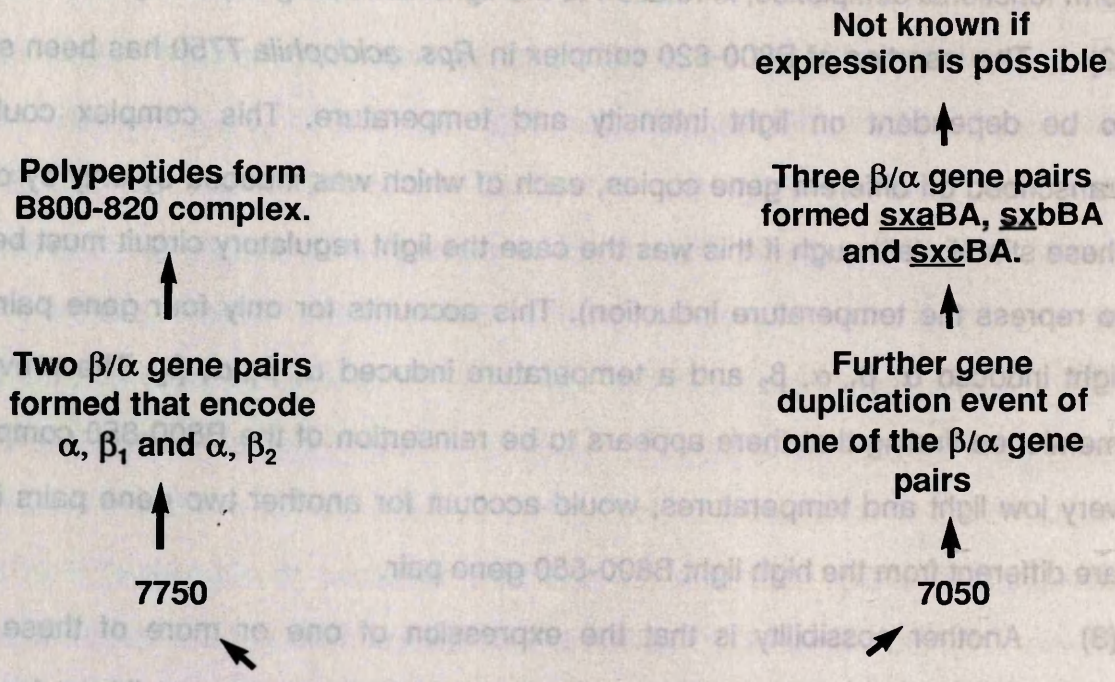
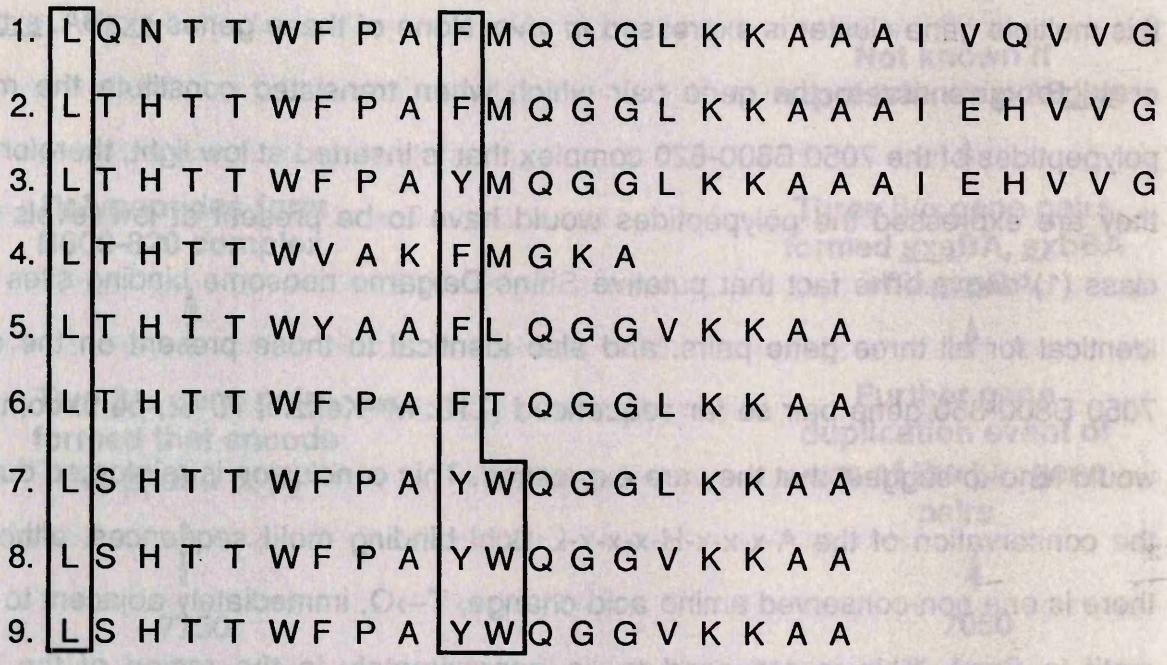



**Figure 9.1:** Hypothetical scheme for the evolution and divergence of the multiple gene cluster sxaBA, sxbBA and sxcBA from 7050, and the analogous genes in 7750 that encode the polypeptides that comprise the B800-820 complex. This scheme allows for two gene pairs in 7750 and that sxbBA is closer to sxcBA than sxaBA.

hypothetical scheme for the evolution of the gene pairs that give rise to this multiple gene cluster and the polypeptides in the 7750 B800-820 complex is suggested in Figure 9.1. At the time of writing experiments were being carried out to determine if this multiple gene cluster is expressed *in vivo*. None of these genes sxaBA, sxbBA or sxcBA give rise to the gene pair which when translated constitute the main polypeptides of the 7050 B800-820 complex that is inserted at low light, therefore, if they are expressed the polypeptides would have to be present at low levels i.e., class (1) above. The fact that putative Shine-Dalgarno ribosome binding sites are identical for all three gene pairs, and also identical to those present on the only 7050 B800-850 gene pair so far sequenced (λ16: M$^{ac}$Kenzie, R. C., pers. comm.), would tend to suggest that they are expressed. This conclusion is reinforced due to the conservation of the A-x-x-x-H-x-x-x-L Bchl binding motif sequences, although there is one non-conserved amino acid change, T→Q, immediately adjacent to this motif in SxaA. This is proposed to lie approximately in the region of the lipid headgroups and may therefore be allowed. Nevertheless, the propensity for random genetic drift in a non-restrained sequence i.e., one that is not being expressed and, therefore, not able to confer a selective advantage, is sufficiently strong that mutations would have been expected to have disrupted at least one of these consensus sequences or the transmembrane hydrophobic domain.

A further interesting feature of SxaA, SxbA and SxcA is the conservation of a methionine residue in all three genes immediately adjacent to the aromatic residue at their C-terminal end. Aromatic residues in this region have been proposed to be important in determining the position of the absorption maxima of the Bchl-dimer in the native complex (see 1.5.2). From Fig. 9.1 and Chapter 8 the translated products of these genes have been shown to be extremely homologous to the polypeptides which comprise the 7750 B800-820 complex. Both SxaA and SxbA exhibit a FM motif whereas SxcA shows YM. The only other example as yet sequenced of the FM motif is the *Rps. palustris* 2.1.6 B800-850 $\alpha_2$ polypeptide. This species produces a B800-850 complex at high and low light but the absorption spectrum and the




(1) SxaA

(2) SxbA.

(3) SxcA

(4) *Rps. palustris* 2.6.1 B800-850$\alpha_2$.

(5) *Rps. acidophila* 7050 B800-820.

(6) *Rps. acidophila* 7750 B800-820.

(7) *Rps. acidophila* 7050 B800-850.

(8) *Rps. acidophila* 7750 B800-850.

(9) *Rps. acidophila* 10050 B800-850.

**Figure 9.2:** Comparison of the published (Zuber, 1990) α-apoprotein C-terminal ends, of peripheral *Rps. acidophila* antenna complexes with those identified in this study. Alignments are shown from the conserved Leucine residue (left box) of the A-x-x-x-H-x-x-x-L structural motif. The right-hand box highlights the aromatic residues of this polypeptide which are proposed to interact with the dimeric Bchl of the complex and determine the precise NIR absorption of this component.

polypeptide composition of each is different. The precise stoichiometry associated with each complex is still uncertain, although presently 4α and 3β polypeptides have been sequenced, with the three other α-polypeptides exhibiting the 'normal' B800-850, high light YW motif. Intriguingly, however, it has been shown (Evans, 1989) that the stoichiometric amount of the B800-850 $\alpha_2$ polypeptide is enhanced at low light relative to high light grown cells. Assuming that SxaBA and SxbBA (and SxcBA) genes can give rise to a functional complex, then the FM motif is in both known cases found associated with a complex that is composed of more types of polypeptides than the usual β/α repeating unit, and is present under low light intensities. Whether this is purely an evolutionary coincidence, or has some real functional significance is as yet unknown.

In conclusion, the challenge proffered from *Rps. acidophila* towards understanding all the facets of photosynthesis is indeed a stimulating one. The multitude of genes, their inter-relationship and co-ordinate regulation under various non-optimal conditions should provide valuable insights, not only with respect to prokaryotic photosynthesis but also to bacterial physiology in general.

# Appendices

## A.1 Compositions of growth media.

**Succinate medium.** (Bose, 1969)

| | per litre. |
|---|---|
| Concentrated base. | 20ml |
| Di-potassium hydrogen orthophosphate (1M). | 10ml |
| Potassium di-hydrogen orthophosphate (1M). | 10ml |
| Ammonium sulphate, (10%). | 5ml |
| Sodium or potassium succinate pH6.8 (1M). | 10ml |
| Growth factors. | 1ml |
| Casamino acids. | 1g |

For minimal Succinate medium the casamino acids were omitted.

| Concentrated base. | per litre. |
|---|---|
| Nitrilotriacetic acid. | 10g |
| Magnesium sulphate. | 14.45g |
| Calcium chloride, $2H_2O$. | 3.4g |
| Ammonium molybdate. | 9.25mg |
| Ferrous sulphate, $7H_2O$ | 99mg |
| Nicotinic acid. | 50mg |
| Aneurine hydrochloride. | 0.5g |
| Biotin. | 0.5mg |
| Metos44 | 50ml |

Add ½ of amount of final volume, pH to 6.8 with 5N KOH, make up to final volume

| Growth factors. | per litre. |
|---|---|
| Biotin. | 20mg |
| Sodium hydrogen carbonate. | 0.5g |
| Add $H_2O$ and dissolve, | 1 (litre) |
| Then add and boil to dissolve: | |
| Nicotinic acid. | 1g |
| Aneurine hydrochloride. | 0.5g |
| 4-Aminobenzoic acid. | 1g |

| Metos 44. | per 100ml |
|---|---|
| EDTA. | 250mg |
| Zinc sulphate. | 1095mg |
| Manganous sulphate, $4H_2O$. | 154mg |
| Copper sulphate, $5H_2O$. | 39.2mg |
| Cobaltous nitrate, $6H_2O$. | 24.8mg |
| Ferrous sulphate, $7H_2O$. | 500mg |
| di-Sodium tetraborate, $10H_2O$. | 17.7mg |
| Concentrated Sulphuric acid. | 2 drops |

**Pfennig's medium.** (Pfennig, 1969) per litre.

| | |
|---|---|
| Potassium di-hydrogen orthophosphate. | 1g |
| Magnesium sulphate, $7H_2O$. | 0.4g |
| Sodium chloride. | 0.4g |
| Calcium chloride. $2H_2O$. | 50mg |
| Sodium succinate. | 1.5g |
| Ammonium chloride. | 0.5g |
| Ferric citrate solution. | 5ml |
| Trace element solution. | 10ml |

For this medium utilising an alternate carbon source:
(20mmols carbon per litre)

| | |
|---|---|
| Sodium pyruvate. | 0.87g |
| Malic acid. | 0.75g |

Ferric citrate solution.

Add 100mg Ferric citrate to 100ml of boiling distilled water.

Trace element solution. per litre.

| | |
|---|---|
| EDTA. | 0.5g |
| Ferrous sulphate, $7H_2O$. | 10mg |
| Manganous chloride, $4H_2O$. | 3mg |
| Boric acid | 30mg |
| Cobalt chloride, $2H_2O$. | 20mg |
| Calcium chloride, $2H_2O$. | 1mg |
| Nickel chloride, $6H_2O$. | 2mg |
| Sodium molybdate, $2H_2O$. | 3mg |

**Medium for *Rps. cryptolactis.*** (Stadtwald-Demchick *et al*, 1990)
Optimal growth temp. 38-40°C

To 500ml deionised water, add, per litre

| | |
|---|---|
| Potassium phosphate buffer (0.64M). | 15ml |
| Ammonium chloride, (10%). | 10ml |
| EDTA, (1%). | 0.5ml |
| Mangnesium sulphate, $7H_2O$ (20%). | 1ml |
| Calcium chloride, $2H_2O$ (7.5%). | 1ml |
| Vitamin $B_{12}$ solution (20µg/ml). | 1ml |
| Niacin (10mg/ml). | 1ml |
| PABA (3mg/ml). | 1ml |
| Sodium sulphite, $5H_2O$. | 0.5ml |
| Sodium pyruvate. | 2.2g |
| True Blue trace element solution. | 1ml |
| Chelated iron solution. | 2ml |

pH to 6.8 and bring final volume to 1l with deionised $H_2O$.

| Potassium phosphate buffer (0.64M). | per litre |
|---|---|
| Di-potassium hydrogen orthophosphate. | 60g |
| Potassium di-hydrogen orthophosphate. | 40g |

pH to 6.8 and bring final volume to 1litre with deionised $H_2O$.

| True Blue trace element solution. | per 200ml |
|---|---|
| EDTA. | 2.5g |
| Manganous chloride, $4H_2O$. | 0.2g |
| Boric acid | 0.1g |
| Sodium molybdate, $2H_2O$. | 0.1g |
| Zinc chloride, $2H_2O$. | 50mg |
| Nickel chloride, $6H_2O$. | 50mg |
| Cobalt chloride, $2H_2O$. | 20mg |
| Cuprous chloride, $2H_2O$. | 10mg |
| Sodium selenate, | 5mg |
| Sodium metavanadate | 5mg |

Bring volume to 250ml.

Chelated iron solution.

To 900ml deionised water, add:

| | |
|---|---|
| EDTA | 2g |
| Ferrous chloride, $4H_2O$. | 1g |
| conc. HCl | 3ml |

Bring volume to 1 litre.

| **Luria-Bertani (LB)Medium.** | per litre. |
|---|---|

To 950ml of distilled $H_2O$, add:

| | |
|---|---|
| Bacto-tryptone. | 10g |
| Bacto-yeast extract. | 5g |
| Sodium chloride | 10g |

Dissolve solutes and bring pH to 7.0 with 5N NaOH. Adjust volume to 1 litre.

When solid media was required for all the above media, 15g/l agar was added prior to autoclaving.

## A.2 Compositions of buffers, stock and hybridisation solutions.

| **TE** | per litre. |
|---|---|
| Tris.Cl pH 8.0 (1M). | 10ml |
| EDTA (0.5M). | 2ml |

Adjust pH as required.

| **50 x TAE.** | per litre. |
|---|---|
| Tris base. | 242g |
| Glacial acetic acid. | 57.1ml |
| EDTA (0.5M, pH 8.0) | 100ml |

| **20 x TBE.** | per litre. |
|---|---|
| Tris base. | 219g |
| Boric acid. | 110g |
| EDTA. | 19g |

pH to 8.3 with boric acid

| **20 x SSC.** | per litre. |
|---|---|

To 800ml, add:

| | |
|---|---|
| Sodium chloride. | 175.3g |
| Sodium citrate | 88.2g |

Adjust pH to 7.0 with 10N NaOH and make up to 1litre.

| **20 x SET.** | per litre. |
|---|---|
| Sodium chloride. | 175.3g |
| EDTA. | 7.4g |
| Tris base | 48.4g |

| **10 x STE.** | per litre. |
|---|---|
| Tris.Cl, pH 8.0 (1M). | 100ml |
| Sodium chloride | 58g |
| EDTA (0.5M) | 20ml |

**100 x Denhardt's solution.**

| | |
|---|---|
| Bovine Serum Albumin (Sigma, Fraction V). | 2% |
| Ficoll. | 2% |
| Polyvinylpyrollidone. | 2% |

**Pre-hybridisation solution.**

4 x SET, 10 x Denhardt's, 0.1% SDS, 0.1% Sodium pyrophosphate, 50µg/ml denatured salmon sperm DNA.

## A.3 Compositions of acrylamide solutions.

| **PAGE Acrylamide stock.** | per 75ml |
|---|---|
| 30% Acrylamide | 75g |
| 0.8% methylene-bis-acrylamide | 2g |

| **SDS-PAGE Running gel buffer.** | per 250ml |
|---|---|
| Tris base. | 45.3g |
| Sodium dodecyl sulphate (SDS) | 1g |

Adjust to pH 8.8

| **SDS-PAGE Stacking gel buffer.** | per 250ml |
|---|---|
| Tris base. | 15.1g |
| Sodium dodecyl sulphate (SDS) | 1g |

Adjust to pH 6.8

| **SDS-PAGE Electrolyte.** | per litre |
|---|---|
| Tris base | 3g |
| Sodium dodecyl sulphate (SDS) | 1g |
| Glycine | 14.6g |

**SDS-PAGE Stacking gel (6%).**

| | |
|---|---|
| Stacking gel buffer. | 3.75ml |
| PAGE Acrylamide stock. | 1.5ml |
| Distilled water | 9.45ml |
| Ammonium sulphate (10%) | 150µl |
| TEMED | 8µl |

| **SDS-PAGE Resolving gradient gel.** | 10% | 20% |
|---|---|---|
| PAGE Acrylamide stock. | 7.5ml | 20ml |
| Running gel buffer. | 6ml | 6ml |
| Distilled water. | 8.7ml | 0.6ml |
| Glycerol (50%). | 1.5ml | 1.5ml |
| Ammonium sulphate (10%). | 90µl | 90µl |
| TEMED. | 8µl | 8µl |

| **Deriphat-PAGE gel buffer.** | per 250ml |
|---|---|
| Tris base | 0.75g |
| Glycine | 3.6g |

| **Deriphat-PAGE resevoir buffer (10x).** | per litre |
|---|---|
| Tris base | 15g |
| Glycine. | 72g |

Adjust pH to 8.3

| **Deriphat-PAGE Electrolyte.** | per 500ml |
|---|---|
| Resevoir buffer (10x). | 50ml |
| Deriphat. | 1g |

| **Deriphat-PAGE Resolving gel.** | for 2mm gel |
|---|---|
| PAGE Acrylamide stock. | 13ml |
| Deriphat gel buffer. | 15ml |
| Deriphat resevoir buffer (10x). | 5.4ml |
| Distilled water. | 26.5ml |
| Ammonium persulphate (10%). | 0.6ml |
| TEMED | 30µl |

| **Sequencing acrylamide stock.** | per 500ml |
|---|---|
| Acrylamide | 96.5g |
| Urea (Ultrapure) | 210g |
| methylene-bis-acrylamide | 3.35g |
| TBE (10x) | 50ml |

Deionise with Amberlite (1g/ml) for 2h, filter and store dark at 4°C.

| **Urea solution (7M).** | per 500ml |
|---|---|
| Urea (Ultrapure) | 210g |
| TBE (10x) | 50ml |

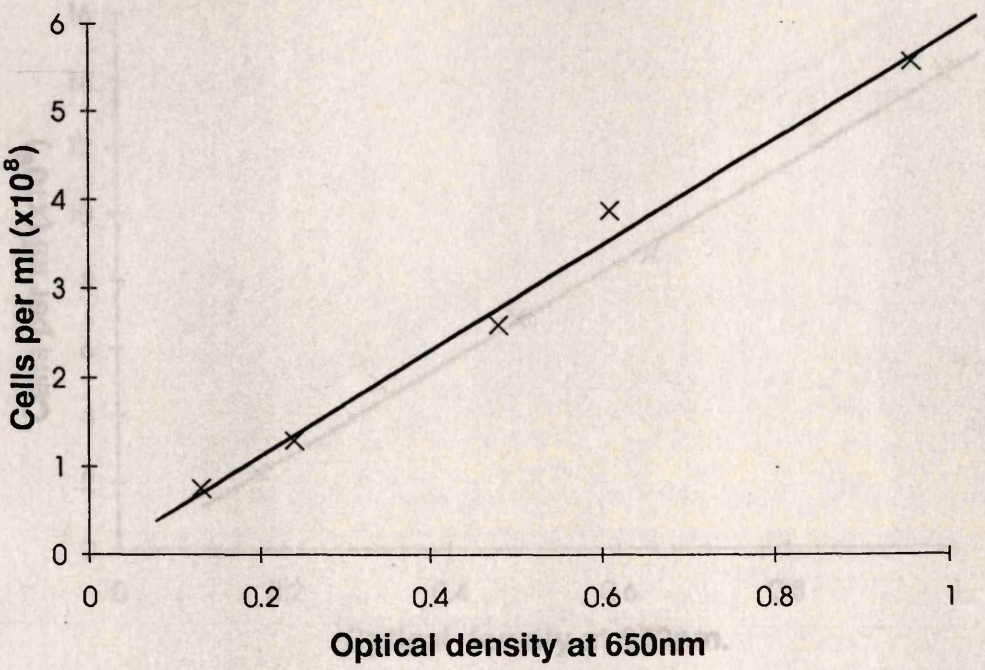
Deionise with Amberlite (1g/ml) for 2h, filter and store dark at 4°C.

**For sequencing gel percentages, mix:**

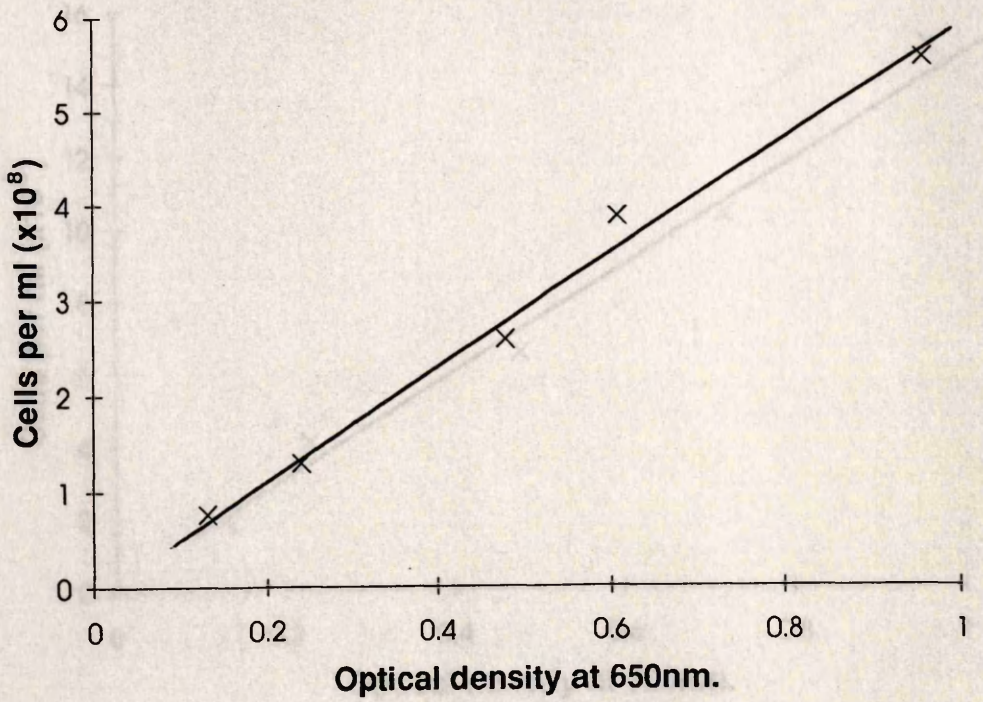
| Percentage | Seq. Acrylamide stock. | Urea solution |
|---|---|---|
| 6 | 24ml | 56ml |
| 8 | 32ml | 48ml |
| 10 | 40ml | 40ml |
| 12 | 48ml | 32ml |

**Graphs of cell numbers versus optical density.**

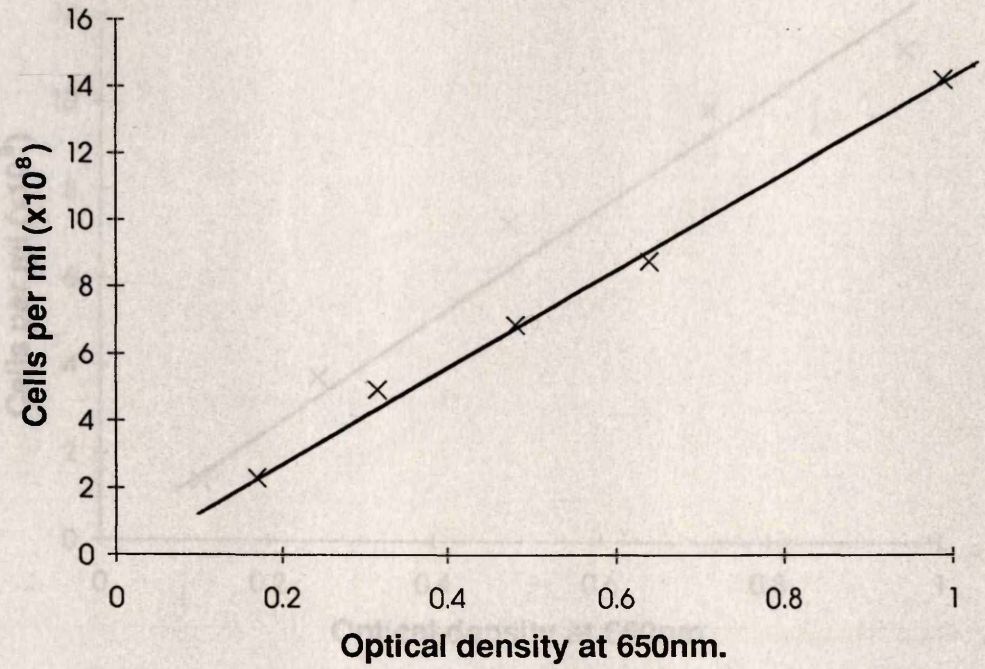
**Rps. acidophila 7750: photoheterotrophic**



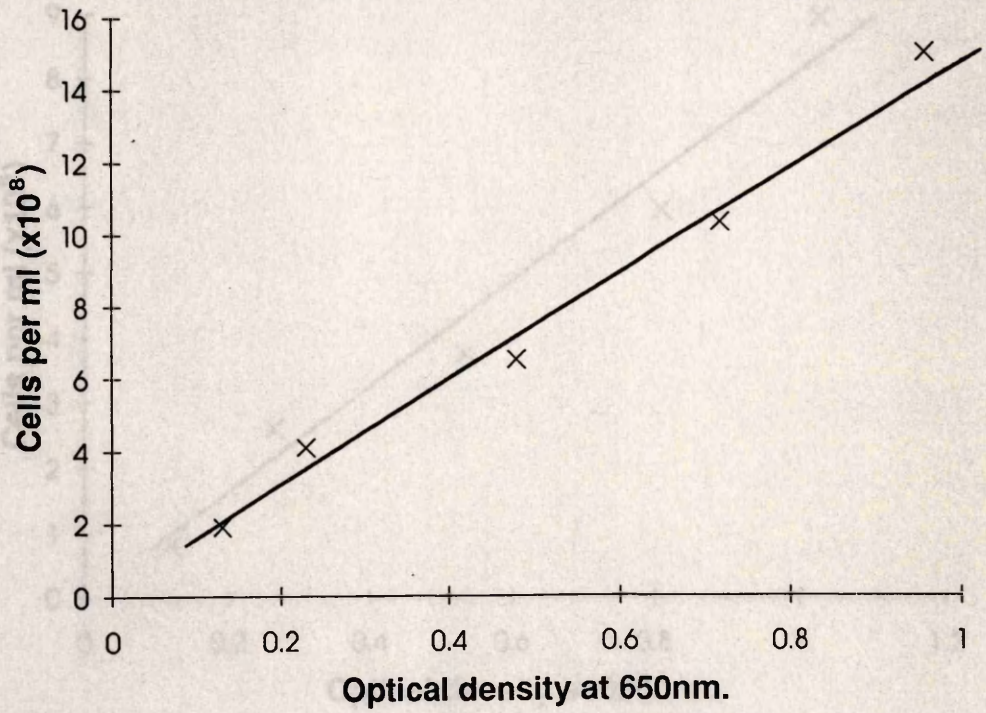

**Rps. acidophila 7750: chemoheterotrophic**



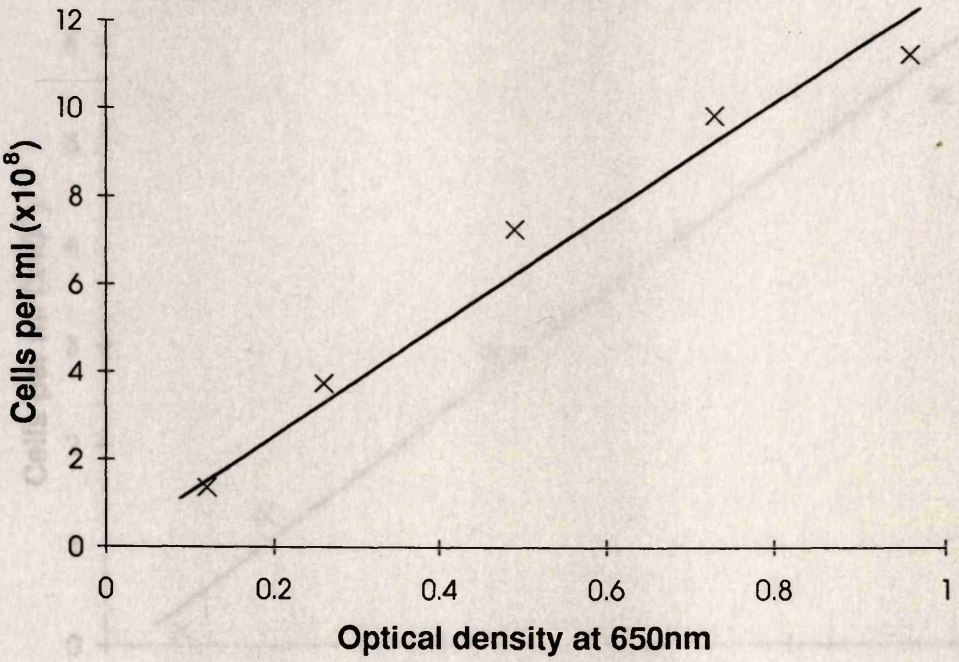
**Rps. palustris 2.1.6: photoheterotrophic**



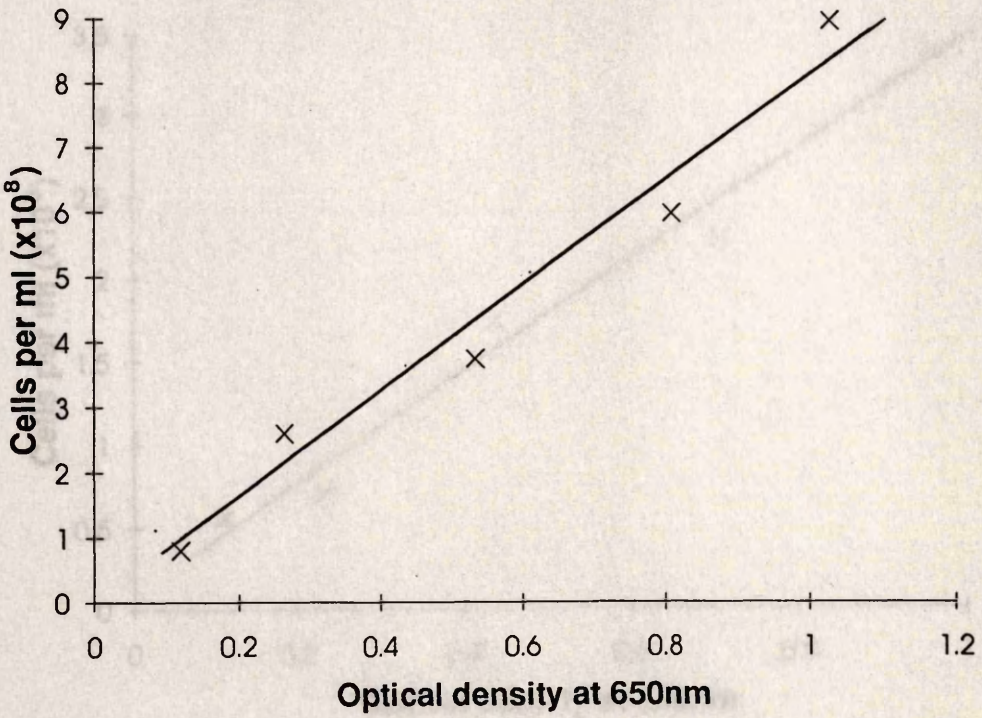

**Rps. palustris 2.1.6: chemoheterotrophic**



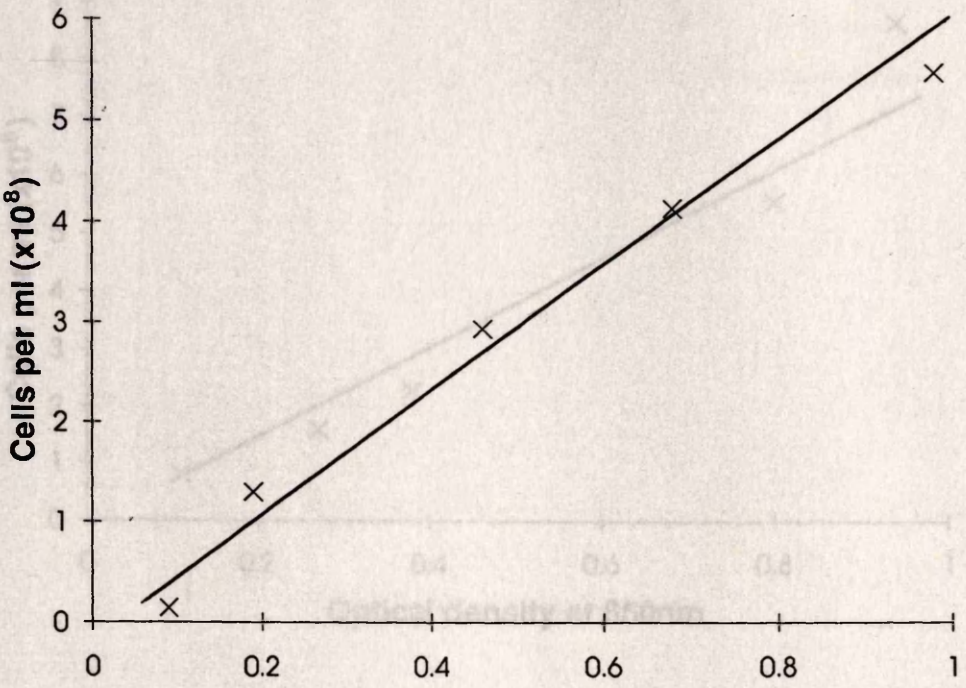
**Rb. sphaeroides 8253: photoheterotrophic**



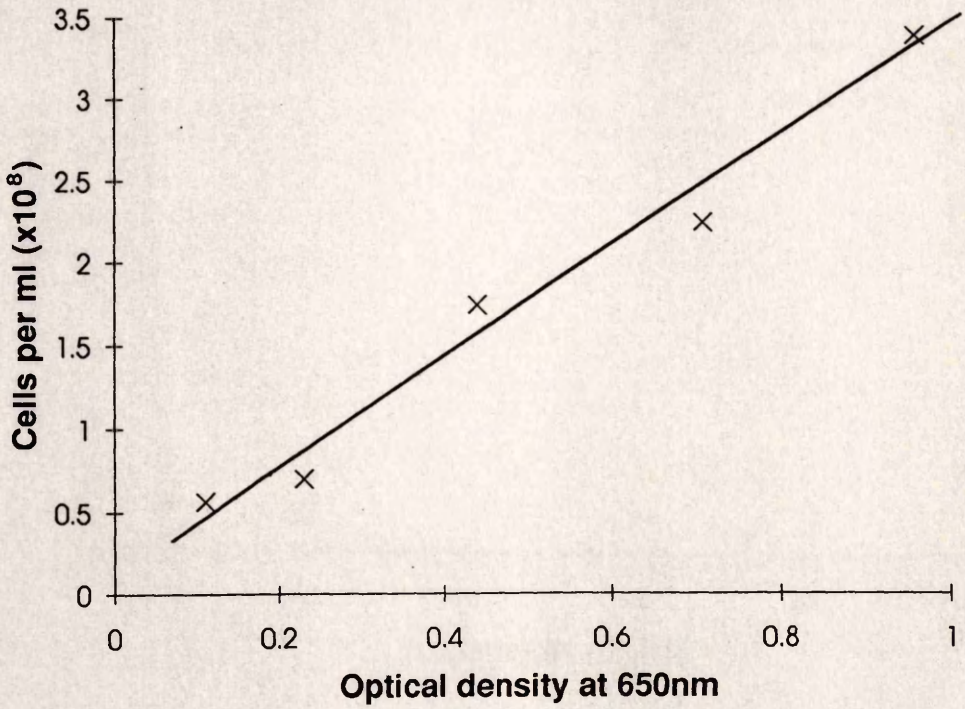

**Rb. sphaeroides 8253: chemoheterotrophic**

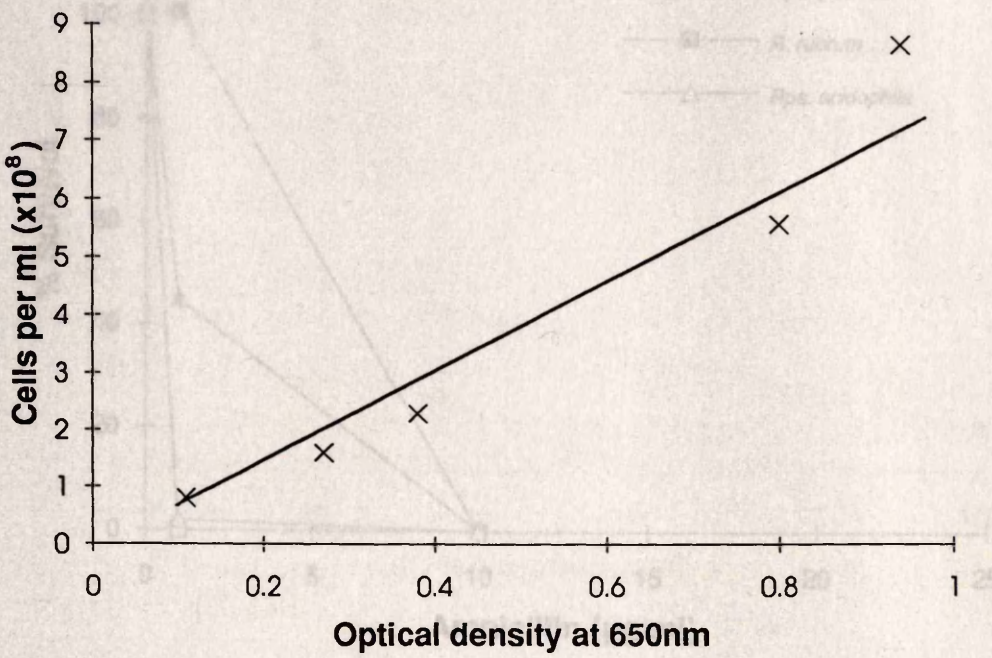


**R. rubrum S1: photoheterotrophic**

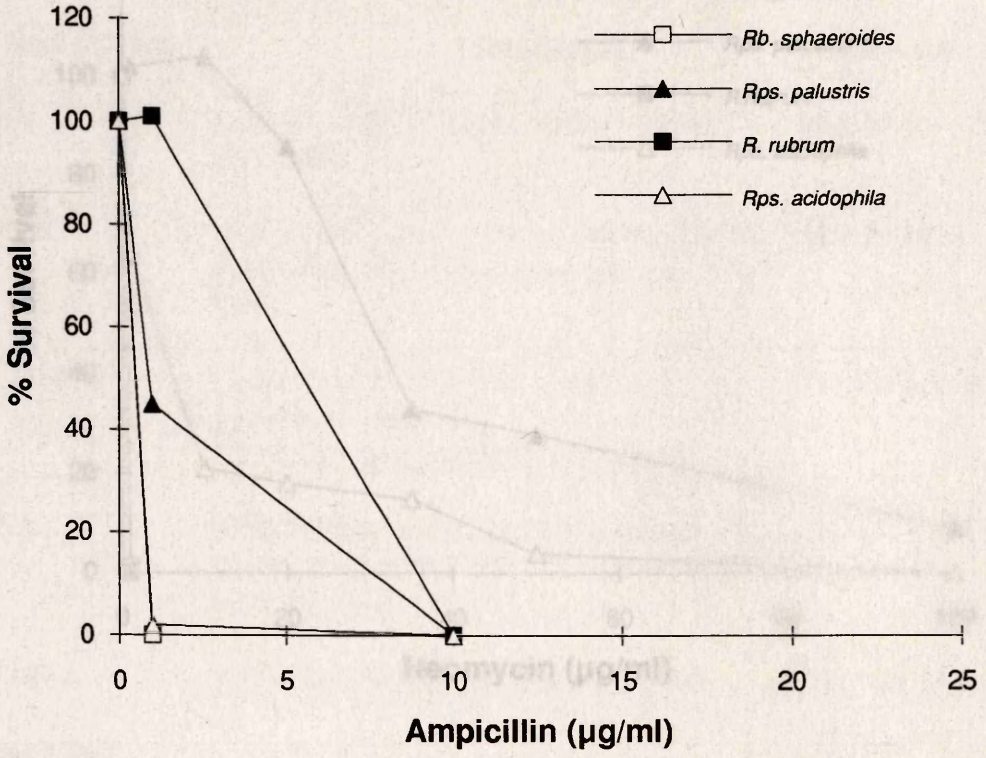


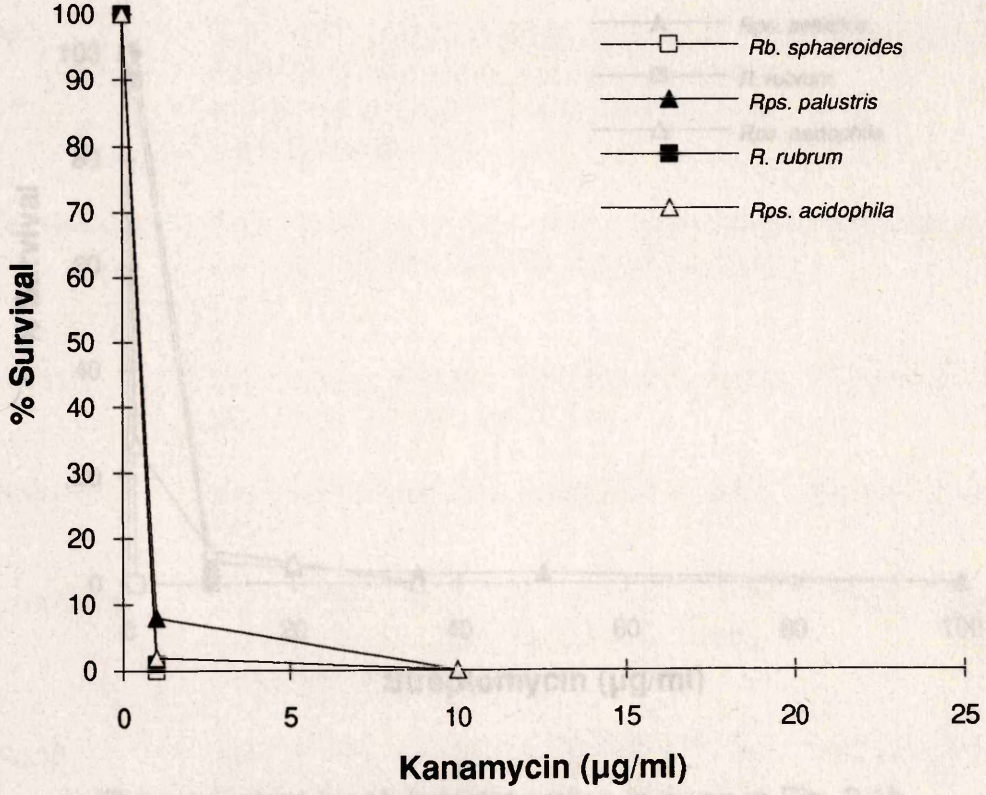

**R. rubrum S1: chemoheterotrophic**

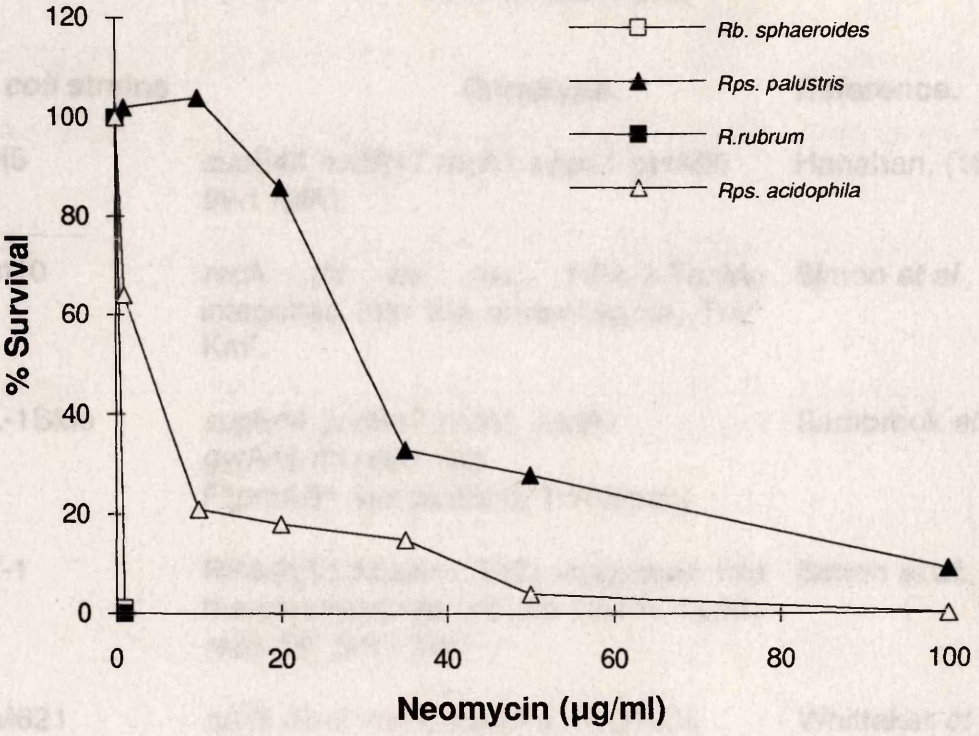


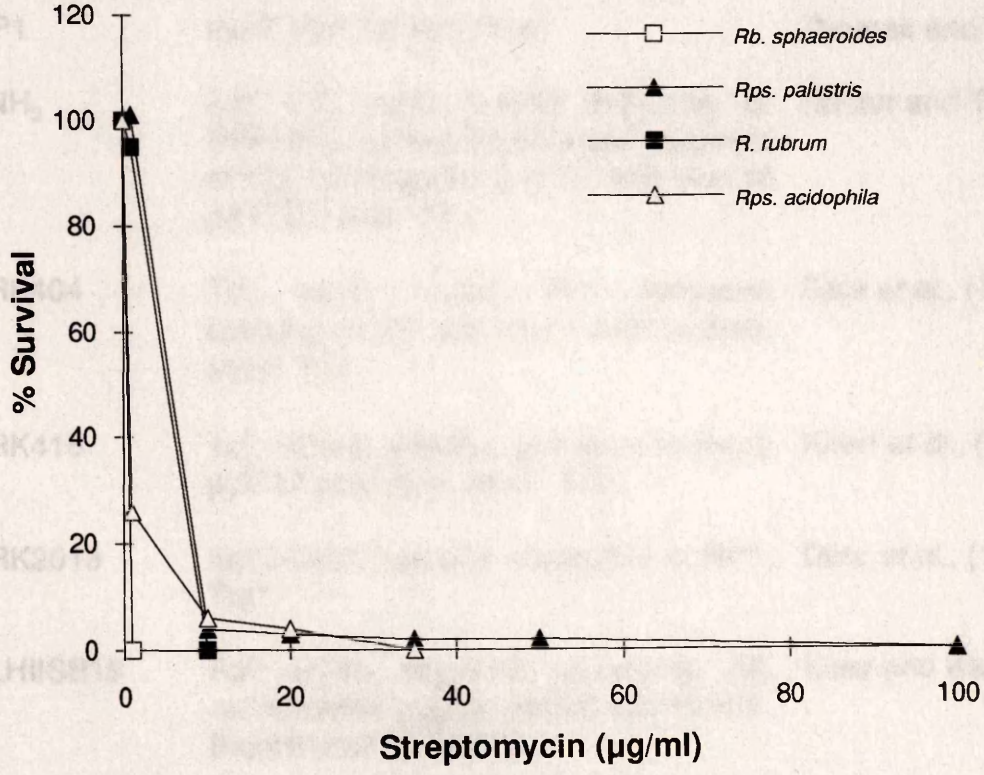

E. coli strain Sm10
Cells per ml (x10⁸)
0
1
2
3
4
5
6
7
8
9
0
0.2
0.4
0.6
0.8
1
Optical density at 650nm

**Rhodospirillaceae susceptibility to antibiotics.**






The equivalent graph for tetracycline is given in Fig. 6.1b.

**Genotypes and relevant characteristics of all *E. coli* strains and plasmids used in this work.**

| ***E. coli* strains** | **Genotype.** | **Reference.** |
|---|---|---|
| DH5 | *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 | Hanahan, (1985). |
| Sm10 | *rec*A *thi thr leu*; RP4-2-Tc::Mu integrated into the chromosome; $Tra^+$ $Km^r$. | Simon *et al.,* (1983) |
| XL-1Blue | *sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A46 *thi rel*A1 *lac*⁻ F'[proAB⁺ *lac*I *lac*ΔM15 Tn10(tetr)] | Sambrook *et al.,* (1989) |
| 17-1 | RP4-2(Tc::Mu,Km::Tn7) integrated into the chromosome; *thi pro hsd* R *hsd*M+ *rec*A $Tp^r$ $Sm^r$, $Tra^+$ | Simon *et al.,* (1983) |
| NM621 | *hsr*R *mcr*A *mcr*B *sup*E44 *rec*D1009 | Whittaker *et al.,* (1988) |
| **Plasmids** | | |
| RP1 | Inc-P; $Ap^r$ $Tc^r$ $Km^r$, $Tra^+$ | Thomas and Smith, (1987) |
| $pNH_2$ | $Sm^r$ $Tc^r$, Inc-Q, 9.45kb derivative of RSF1010; 8.05kb EcoRI-AvaI fragment of RSF1010 ligated to a $Tc^r$ fragment of pAT153, $Mob^+$ $Tra^-$. | Hunter and Turner, (1988). |
| pRK404 | $Tc^r$; Inc-P, 10.6kb RP1 derivative carrying pUC9 polylinker and *lac*Z(α), $Mob^+$ $Tra^-$. | Ditta *et al.,* (1985). |
| pRK415 | $Tc^r$; 10.5kb pRK404 derivative carrying pUC19 polylinker, $Mob^+$ $Tra^-$. | Keen *et al.,* (1988) |
| pRK2013 | $Km^r$; ColE1 replicon, derivative of RP1, $Tra^+$ | Ditta *et al.,* (1985) |
| pLHIISB18 | $Ap^r$; 0.7kb fragment containing *Rb. sphaeroides* puc BA cloned into HincII-, BamHI sites of pUC18 | Kiley and Kaplan, (1987) |
| pKan2 | $Ap^r$; 3.3kb HindIII fragment of Tn5 cloned into pBR322. | Hunter, (1988) |

| | | |
|---|---|---|
| pSUP202 | $Ap^r$ $Tc^r$ $Cm^r$; $Mob^+$ $Tra^-$: ColE1 replicon. | Simon *et al.*, (1983) |
| pSUP2021 | pSUP202::Tn5; $Km^r$. | Simon *et al.*, (1983) |
| pBluescript II $SK^-$ | $Ap^r$; 2.96kb phagemid *lacZ* ColE1 replicon. | Stratagene, Cambridge, UK. |
| pB52 | $Ap^r$; 2.1kb SalI *Rps. acidophila* fragment, containing sxaA, sxbBAand sxcBA cloned into pBluescript II $SK^-$ | This work. |
| pRKB52 | $Tc^r$; 2.1kb HindIII-KpnI fragment of pB52 containing sxaA, sxbBAand sxcBA cloned into pRK415. | This work. |

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