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Towards
the Mechanism of
Carotenogenesis
in Myxococcus xanthus.

by
Paul Russell Henry Robson

Thesis submitted for the degree of Doctor of Philosophy
in the University of Warwick
Department of Biological Sciences

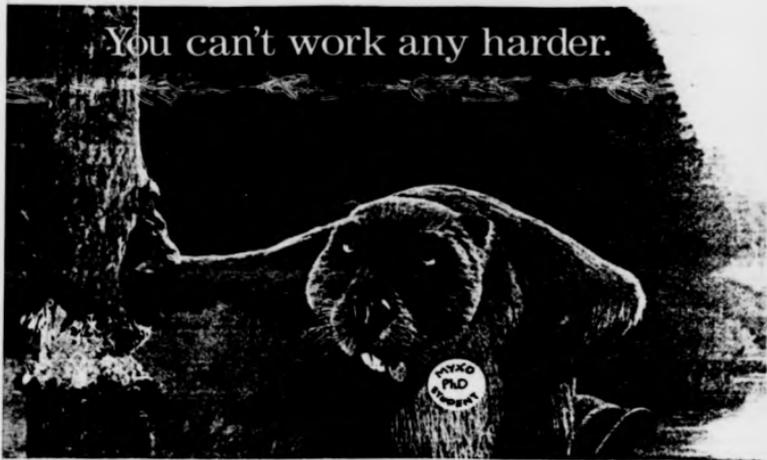
November 1981

NUMEROUS ORIGINALS
IN COLOUR



For the Robson clan

You can't work any harder.



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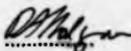
DECLARATION

This thesis is my own work unless otherwise acknowledged and at no time has been submitted for another degree

A handwritten signature in black ink, appearing to read 'P. Robson', written over a horizontal dotted line.

P. Robson

I certify that this statement is correct

A handwritten signature in black ink, appearing to read 'D. Hodgson', written over a horizontal dotted line.

D. Hodgson

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I thank you all, goodnight.

ABBREVIATIONS.

(B)Chl; (bacterio)chlorophyll

(B)Chl^{*}; excited state (bacterio)chlorophyll

Car; carotenoid

Car^{*}; excited state carotenoid

Car^c; constitutive expression of carotenoids

Car⁻; unable to synthesise carotenoids

carQRS; region of DNA encoding the carQ carR and carS genes

DN; defined nutrient medium

d-ALA; delta-aminolevulinic acid

DABCO; 1,4-diazobicyclo [2.2.2.] octane

GGDP; geranyl geranyl diphosphate

IDP; inosine di-phosphate

kJ; kilo-joule

lacZ; gene encoding beta-galactosidase

LH; light harvesting

LWUV; long-wave ultra-violet; SWUV; short wave UV

MVA; mevalonic acid

nm; nanometres

³O₂; molecular oxygen

¹O₂; singlet excited state oxygen

pO₂; partial pressure of oxygen

p^{QRS}; light inducible promoter controlling expression of carQRS region

p^X; un-characterised promoter sequence upstream to p^{QRS} active in E. coli

microE m⁻² s⁻¹; micro Einsteins per metre squared per second

RC; reaction centre

tet^r; tetracycline resistance phenotype

SUMMARY.

Mycrococcus xanthus is a Gram negative, heterotrophic, soil dwelling, bacterium. It exhibits a number of interesting characteristics, including the synthesis of carotenoids. Carotenoids protect the cells from lethal photolysis by protoporphyrin IX in the presence of light (Burchard and Dworking, 1988, J.Bac., 87, 1165-1188). The region of DNA which encodes the proteins responsible for the control of carotenogenesis by light has been identified (Martinez-Laborda and Murillo, 1989, Genetics, 122, 481-490, Hodgson, manuscript in preparation). This region, termed carQRS, is under the control of a light inducible promoter, termed p_{QRS}. The aim of this study were to determine the mechanism by which light regulates p_{QRS}, and to establish its possible application to biotechnology by reconstituting the light inducible mechanism in a heterologous host, Escherichia coli. To facilitate these studies the p_{QRS} promoter was linked to a promoter-less lacZ gene, allowing a simple assay for promoter activity.

The p_{QRS} region exhibits two expression patterns in E. coli which depend on the size of the p_{QRS} region studied. Only one of these is believed to be the expression of p_{QRS}, showing a low level of expression which may be increased in the presence of the carQRS region. The second expression pattern is believed due to a promoter-like region upstream of p_{QRS}, termed p'. An E. coli strain permeable to protoporphyrin IX has been isolated. This strain exhibits photolysis, in the presence of protoporphyrin IX, which may be quenched by carotenoids, and thus is an ideal system for further studies of the induction of p_{QRS} in E. coli.

The p_{QRS} promoter has been extensively studied in its native host. Genetic studies in which a mutant of M. xanthus deleted for carQRS was reconstituted with fragments of the region showed an open reading frame previously without function was required for full expression from p_{QRS}.

Light induction of batch cultures of M. xanthus was used to study in part the physiology of the inductive process. The level to which p_{QRS} activity is induced by light decreases throughout the growth cycle, and is subject to feedback control by endogenous carotenoids. The p_{QRS} promoter may be induced by defined laser light of wavelength 410nm, the maxima of absorption by protoporphyrin IX. Extensive continuous culture experiments showed that the p_{QRS} promoter may be induced in the dark by increasing the oxygenation of the culture. Typically two-fold induction was seen. In addition it was shown that the level of oxygenation determined the extent to which light stimulates expression from p_{QRS}. A further effect of oxygen on the accumulation of carotenoids was noted, this was not dependent on p_{QRS} activity. Under steady state conditions in which the p_{QRS} promoter was activated by light, increasing oxygenation was shown to alter the type of carotenoids which accumulated without altering the level of expression from p_{QRS}.

The reactive species involved in the stimulation of p_{QRS} activity by light and oxygen was investigated. The promoter could not be induced by exogenous hydrogen peroxide, or by the presence of methyl viologen, a superoxide radical generator. However, it was shown p_{QRS} the photoactive dye, Toluidine Blue O, could induce expression from p_{QRS} under red light stimulation which does not stimulate the native photoreceptor. This induction was shown to be due to singlet oxygen by p_{QRS} singlet oxygen quencher. Additionally it was shown that induction of p_{QRS} by the native photoreceptor could be quenched in the same way. Thus it has been shown that light induces carotenogenesis in M. xanthus through singlet oxygen.

1. INTRODUCTION.

1.A. Myxobacterales

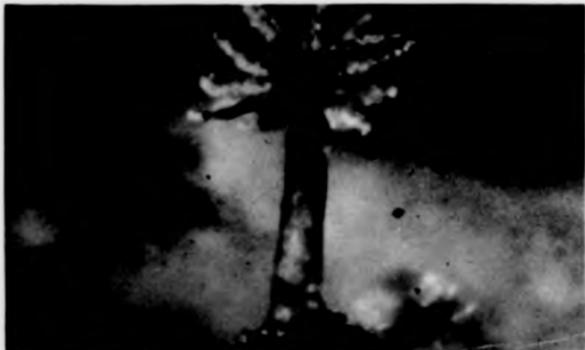
1.A.1. Description of Myxobacteria.

Myxobacteria are Gram negative, rod shaped, soil dwelling, heterotrophs. Their existence was first noted in the Botanical Gazette when in 1892, Roland Thaxter described his observations of a "bright orange-coloured growth occurring upon decaying wood, fungi and similar substances." His Victorian notes detailed many of the interesting features of this genus, which to date still provide an impetus to research groups worldwide.

Much work has concentrated on the most immediately striking aspect of some members of the genus, namely their ability to form elaborate multicellular fruiting bodies. These are produced in response to starvation conditions which trigger a reversal of the outward movement of the swarm of myxobacteria which then glide inwards and accumulate in mounds, or fruiting bodies. Here cells differentiate into myxospores. The myxospore is a physiologically dormant cell serving as a survival and dispersal mechanism (Dworkin and Manoil, 1979). Myxospores are not endospores, being formed from the entire cell, and are resistant to ultraviolet light, heat, and desiccation (Sudo and Dworkin, 1989). The fruiting bodies vary in complexity between, and are characteristic of, different species of myxobacteria. In their most elaborate forms, seen in Chondromyces, and Stigmatella species, they consist of a stalk topped by several sporangioles, often on additional stalks; some examples of fruiting bodies seen in species of Myxobacterales are shown in Figure 1.

Figure 1. Examples of the fruiting body structures seen in species of Myxobacterales: (A) Chondromyces apiculatus; (B) Stigmatella aurantiaca; (C) Myxococcus fulvus; (D) Myxococcus stipitatus. (H.Reichenbach pers. comm.)

A



B



Figure 1.

C



D



Figure 1. (continued)

Myxobacteria feed by grazing on bacteria and fungi. In some cases cellulose can be used, but more usually proteinaceous material is the major source of energy and catabolites. They release an arsenal of degradative enzymes and antibiotics (Hart and Zahler, 1966; Sudo and Dworkin, 1972), one of which, myxobactin, is currently being developed towards commercial ends (Reichenbach et al., 1988).

Myxobacteria exhibit an unusual type of motility known as gliding. This is variously defined as translocation in which no wriggling, contraction, or peristaltic alterations are seen, and which occurs in the absence of obvious locomotory organelles, such as flagella. This type of movement is associated with the presence of extracellular slime and a tendency to form swarms and multicellular patterns (Dworkin and Kaiser, 1981). The swarms may increase the efficiency of lysis of proteinaceous material by myxobacteria in a "wolf-pack" effect. M. xanthus has been shown to exhibit this effect when grown on complex media (Rosenberg et al., 1977). It was shown that cells exhibited increasing growth rates as a function of increasing cell numbers.

In nature, myxobacteria occur in large numbers in the soil and in particular on rotting material or faeces (Singh, 1947). They were originally grown on dung pellets or bacterial cultures, however, several artificial media are now available. A complex medium, in which doubling times of 3.5 hours may be achieved has been devised (Dworkin, 1982); a variation on this media is used in this study (Methods 2.2.4.). In addition a defined medium (A1) has been developed (Methods 2.2.4.); this media reduces the tendency, seen in richer media, whereby M. xanthus lyse at a high frequency (Bretscher and Kaiser, 1978).

M. xanthus displays a number of different pigmentation phenotypes.

The synthesis of carotenoids will be discussed in more detail later. In brief, light induces the synthesis of a number of coloured carotenoids some of which appear unique to myxobacteria (Reichenbach and Kleinig, 1971). The presence of these carotenoids serves to protect the cell from the potentially lethal effects that light may have on biological systems. In addition to the synthesis of carotenoids, M. xanthus displays an unstable pigmentation phenotype. Colonies of M. xanthus may have either a tan or a yellow phenotype. The yellow colouration is due to pigment(s) with an absorption maximum of 379nm (Burchard et al., 1977). In most strains the yellow is the more stable phenotype, a tan culture will often give rise to yellow variants, for instance M. xanthus strain FB (Tan) produces yellow colonies with a frequency of up to 20%. Colonies show an additional phase variation in that tan colonies exhibit a non-swarming phenotype, whereas yellow colonies exhibit a swarming phenotype. The significance of phase variation in M. xanthus remains to be determined.

A number of techniques have been developed which greatly facilitate the study of M. xanthus. Historically no indigenous plasmids have been isolated from M. xanthus, and transformations are unreliable. Generalized transducing phage have been isolated (Campos et al., 1978; Martin et al., 1978). Kaiser and Dworkin (1975) showed that the broad host range phage, P1, adsorbed to M. xanthus and injected its DNA but was not maintained within the cell. Kuner and Kaiser (1981) subsequently showed that the transposon Tn5 may be introduced into M. xanthus by phage P1, and renders the recipient cell kanamycin resistant. This provided a marker to mutate and isolate genes of interest. Shinketa et al. (1983) described a means of reintroducing DNA isolated from M. xanthus to E. coli back in to M. xanthus. This allowed the manipulation of genes of interest to be

carried out in E. coli before reintroduction into the host cell. The region of DNA required for efficient packaging in to phage P1 (P1 inc) has been isolated. A schematic for the introduction of DNA on a plasmid is shown in Figure 2. A hypothetical transposon is integrated close to a Sal I site on a plasmid borne region of the myxobacterial chromosome. The plasmid is introduced into the cell via P1 transduction, the plasmid cannot be stably maintained, however the transposon may integrate into the host chromosome via one of two possible recombinational events. The double crossover results in gene replacement. A single crossover results in the formation of a merodiploid which in addition to homologous regions contains plasmid sequences integrated into the chromosome.

The above techniques have been expanded in a number of ways. A Tn5 lac gene fusion has been developed which allows the assay of expression of the gene into which it has inserted (Kroos and Kaiser, 1984). To facilitate complementation studies between transposon mutated genes, a Tn5 replacement vector (Tn5-132) has been developed (Avery and Kaiser, 1983) which encodes tetracycline resistance. This allows the replacement of an existing Tn5 with the alternative resistance, allowing the more strict kanamycin selection for transduction of a second tagged gene, or the introduction of plasmids which are selected for by kanamycin.

1.A.2. Previous studies of carotenogenesis in Myxococcus xanthus.

It is to Thaxter's "bright orange colour" that this study turns. This colouration is due to the presence of carotenoids whose role within the myxobacterial cell was first investigated after a chance observation of M. xanthus strain F.B. While attempting to isolate phage on lawns of M.

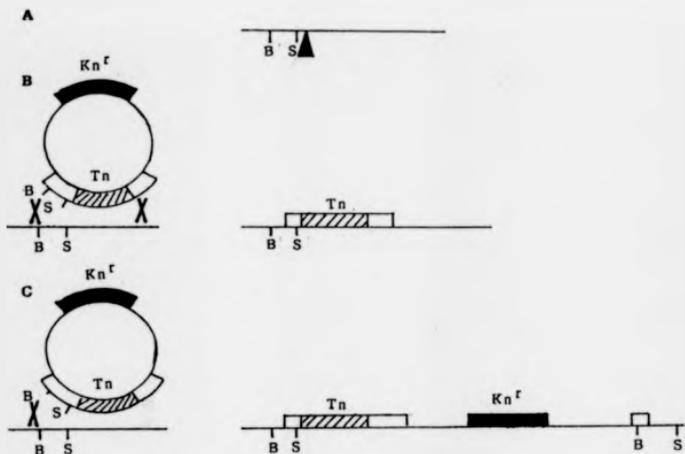


Figure 2. A diagrammatic representation of the integration of plasmid DNA into the *M. xanthus* chromosome by homologous recombination: A) shows the host chromosome and the point at which the transposon is inserted into the host DNA present on the vector. B) shows the double crossover in which the fragment of DNA on the plasmid in which the transposon is inserted replaces the homologous piece of DNA in the chromosome. This chromosomal piece is thus lost. C) shows the production of a merodiploid in which a single crossover has occurred. The plasmid has become integrated into the chromosome and there are now two copies of the region of DNA present on the plasmid, one of which contains the transposon. B - Bam HI; S = Sac I. Adapted from O'Connor and Zusman (1983).

xanthus R.P. Burchard noticed zones of lysis appearing on old plates discarded on the bench. These "plaques" did not yield active phage and were eventually shown to be areas of photolysis caused by direct sunlight (M. Dworkin, pers. comm.). It was noted that light not only causes photolysis, but in addition stimulated a protective response in the form of the synthesis of carotenoids (Burchard and Dworkin, 1988; Burchard et al., 1988; Burchard and Hendricks, 1989). During batch culture, cells illuminated through-out their growth cycle accumulated carotenoids as they entered the post-log-phase. Both log-phase, and post-log-phase cells when grown under illumination were found to be photoresistant. Dark grown cells, however, did not accumulate this pigment. These dark grown cells were sensitive to light in the lag phase, when their poor physiological state inhibits the expression of an effective carotenogenic response. In addition the proposed photosensitizer, discussed below, accumulates maximally in lag phase, which may contribute to the enhanced sensitivity.

Action spectra were produced for the induction of carotenogenesis, and for the photolysis of cells made deficient in carotenoids (Burchard et al., 1988; Burchard and Hendricks, 1989). The spectra indicated that a porphyrin molecule was acting as the photoreceptor/sensitizer in both phenomena. A porphyrin was isolated, whose intracellular concentration increased in tandem with the increase in photosensitivity, seen towards the end of the growth cycle. Post-log-phase cells contained upto sixteen times as much of the porphyrin as log phase cells. Addition of this porphyrin, or the extract from a late-log-phase culture, to an otherwise non-sensitive (dark grown) culture, made it light-sensitive. The porphyrin molecule was identified as Protoporphyrin IX, a planar tetrapyrrole, which forms part of the heme synthesis pathway prior to the addition of iron. Burchard et al.,

(1966) considered that the photosensitizer resided in the membrane and that this was the site of light induced damage.

The carotenoids present in species of Myxobacterales and the genes involved in carotenogenesis by M. xanthus have been studied. These studies will be addressed after a general overview of carotenoids, their function, and regulation.

It is proposed that the functions and biochemistry of carotenoids involve effects of both light and oxygen. As such it is prudent to review both these phenomena, and their effects on biological systems at this stage.

1.B. Photobiology.

1.B.1. Light Effects on Biological Systems.

The electromagnetic spectrum spans a huge range of wavelengths, from cosmic rays at 0.0001 Angstroms, to electric waves at 100 kilometres. Visible light makes up only a small proportion of this spectrum spanning from around wavelengths of 380nm to 760nm. Ultraviolet radiation below 300nm is mainly absorbed by atmospheric ozone, and infra-red radiation beyond 800nm is mainly absorbed by atmospheric water vapour. Many phenomena in photobiology are adapted to the ultra-violet, and the visible region of light. The spectrum of solar radiation that reaches the Earth's surface has a maxima at around 500nm and it is around this point that photobiological phenomena cluster (Woiken, 1975).

Light controls a variety of biological phenomena. These include phototropism, photosynthesis, timing of flowering, sexual cycles in animals, photoprotection. Light affects aspects of behaviour ranging from the

migratory response of birds to the breeding season of mammals (Scott, 1972). These responses are affected by variation in day length, which can be reproduced under laboratory conditions. Even the sexual cycles of continuous breeders, such as man, have been shown to peak seasonally at around May/June in the northern hemisphere, and correspondingly 6 months later in the southern hemisphere. Insect dormancy (diapause) is also affected by day length, this response, and possibly others too, are modulated by additional environmental factors, such as temperature. Through the action of phytochrome, and the so-called cryptochrome blue/UV receptor, a large number of aspects of plant development are affected by light. These include de-etiolation, leaf movements, photocontrol of enzyme levels and chloroplast development, perception of neighbouring plants, and timing of flowering (Smith, 1978). Light responses in protozoa have received considerable attention. One common response is phototaxis. Euglena show positive phototaxis up to a threshold intensity which if exceeded converts the response into a negative phototaxis. It has been shown that carotenoids are essential in orientating the Euglena relative to the light source (Song and Walker, 1981). Some of the photoresponses of prokaryotes shall be discussed later, others include for instance phototaxis (Hader, 1987), and the visible photo-activation of the DNA repair enzymes responsible for the repair of UV induced DNA lesions (Hanawatt et al., 1979).

1.B.2. Photochemistry.

There are two basic laws of photochemistry which need to be addressed. The first is Einstein's law of photochemistry. This law illustrates

that one quantum of light can bring about a direct primary photochemical change in exactly one molecule of matter, i.e. that all the energy of a single photon may be transferred to a single electron. The second is the Pauli exclusion principle. This law states that in a ground state the spins of two paired electrons are antiparallel (opposite) (Hader and Tevini, 1988).

It is of interest to note that a single photon may be sufficient to bring about a biological response as exemplified by the study of phototaxis in halobacteria (Marwan et al., 1988), and in the unicellular green alga Chlamydomonas reinhardtii (Hegemann and Marwan, 1988). Using Poisson analysis of the effect of light it was shown that these responses can be generated by a single photon.

For light to have an effect on a biological system, the latter must contain molecules which have the correct electronic configuration to absorb this light. Most molecules which occur in the cell, such as water, protein, nucleic acids, lipids, carbohydrates, and their derivatives cannot be stimulated by visible light. However, some molecules, namely (bacterio)chlorophyll, carotenoids, flavines, and porphyrins, contain the extended electron systems required for light stimulation.

The electrons in a molecule are arranged into orbitals, with low energy electrons occupying orbitals close to the nucleus, high energy electrons occupying orbitals further away. When the electrons are occupying their standard orbitals, the molecule is said to be in its ground state. Movement of an electron from one orbital to an orbital further away from the nucleus requires energy - this energy may be provided by a number of means, including the absorption of a photon of light. An

electron thus promoted to an outer orbital results in an excited state molecule (Wolken, 1975).

Electrons may only occupy distinct energy levels (orbitals), intermediate energy levels are "forbidden". Therefore only a specific amount of energy, i.e. only specific quanta of light, may promote an electron to a higher orbital. The energy of light may be measured in electron-Volts (eV), and is proportional to the frequency, and inversely proportional to the wavelength. Hence, from broad spectrum i.e. white light, only specific quanta i.e. specific wavelengths, will be absorbed by a given molecule. This phenomena explains why molecules have a characteristic absorption spectra which may therefore be used to identify a certain type of molecule by the wavelengths it absorbs. Consequently action spectra may be interpreted in that the wavelengths initiating the response allow the identification of the molecule which causes the response.

The two molecular states which shall be considered later are the singlet, and the triplet state. These states may be explained by considering the electronic configuration of a molecule. As previously stated electrons occupy distinct orbitals. Electrons occupying the same orbital have opposite spin. This configuration is said to be the singlet state. If one of the antiparallel electrons is promoted to a higher orbital, and no alteration in the spin of the electron occurs, the molecule is said to be in an excited singlet state. However, if one electron inverts its direction of spin the molecule is said to be in the triplet state (Hader and Tavini, 1987).

There are four types of processes by which an excited state molecule may be returned to either its ground state, or an intermediate state: 1) Radiative Transition, the electron may return to the low energy orbital

with the emission of light (fluorescence); 2) Non-radiative Transition, the electron may return to the low energy orbital without emission of light and without chemical reaction; 3) Intersystem crossing, the electronic excitation energy is transferred to lower energy orbitals, forming a species of lower energy but higher stability and; 4) Chemical Reaction (Wolken, 1975).

The various states described above may be displayed for clarity on a Jablonski diagram (Figure 3), which shows molecular orbitals as horizontal bars at different eV values (y-axis). Electronic excitation and transitions 1) 2) 3) and 4) described above, are illustrated as arrowed lines linking molecular orbitals illustrating the increase or decrease in the energy of the electron. The direction of spin of the electron is shown in the boxes which represent molecular orbitals.

1.B.3. Porphyrins as Photoreceptors and Photosensitizers.

The proposed photoreceptor/sensitizer in M. xanthus is the Protoporphyrin IX molecule (Burchard et al., 1986). This is the precursor to both heme and chlorophyll. To produce heme and its derivatives an Iron molecule is added to the pyrrole ring, to produce chlorophyll, a magnesium molecule is added. In recent years an impetus to research into porphyrin photosensitized photobiology has been provided by the discovery that certain porphyrins target cancer cells, and therefore may be used to selectively photo-lyse these cells (Kessel, 1984). In addition several naturally occurring porphyrins have been implicated as the photoreceptors in a number of photodynamic effects (Becerril and Duke, 1989; Burchard and Dworkin, 1988; Harrison Jr, 1987; Kjeldstad and Johnsson, 1988; Siefertmann-

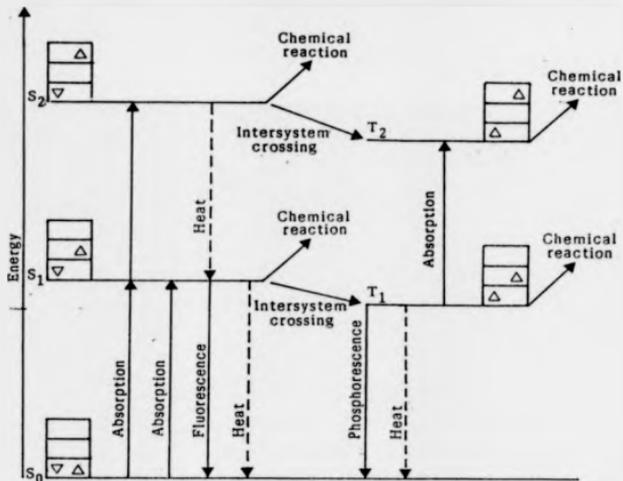


Figure 3. Jablonski diagram showing transitions between singlet and triplet molecular states. S_0 = ground state; S_1 , S_2 = excited singlet states; T_1 , T_2 = excited triplet states. Absorbed energy may be released as fluorescence, phosphorescence, or as a radiation-less conversion (heat). (Δ) designate the direction of the electron spin within one of the three molecular orbitals shown. Taken in part from Hader and Tevini (1987), and Wolken (1975).

Harms, 1987).

Many photodynamic phenomena sensitized by porphyrins involve the participation of molecular oxygen in the generation of reactive oxygen species. Such reactive oxygen species are potentially damaging to cellular systems, however, due to the unusual configuration of molecular oxygen they also have an essential part in the chemistry of the cell. The ground state of molecular oxygen has two unpaired electrons with parallel spin; this triplet state is very stable and restricts the reaction of oxygen with most other molecules. As such the molecular oxygen (or di-oxygen) molecule is unusual in that its singlet state is more reactive than its triplet state (Knox and Dodge, 1985). For reaction to occur the spin restriction must be overcome (Pauli exclusion principle). This is achieved in the formation of a number of reactive oxygen species. Reduction leads to the superoxide anion, hydroxyl radical, and hydrogen peroxide. The hydroxyl radical has been proposed as a major toxic species, however its universal reactivity means it is unlikely to escape indiscriminate scavenging by the numerous potential reactants present in biological systems.

Electronic excitation of molecular oxygen leads to the production of the singlet oxygen molecule. The singlet state is mainly generated by energy transfer from photoexcited species, however, it may result from the action of certain enzymes. The lifetime of the singlet oxygen molecule is solvent dependent, varying from 2-4 micro-seconds in water to 25-100 micro-seconds in polar solvents. Of particular relevance to this study is the observation by Suwa *et al* (1977) that the lifetime of singlet oxygen is increased in the hydrophobic interior of membranes. The singlet oxygen molecule is not indiscriminantly reactive (Kellogg III, 1975), its electrophilic nature results in reaction with unsaturated molecules being

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favoured. The molecule may be quenched by specific amino acids eg. histidine, methionine, and tryptophan and may initiate membrane peroxidation. This results in there being specific cellular targets for the action of singlet oxygen (Knox and Dodge, 1985).

Absorption of a photon of light by a porphyrin molecule results in the generation of a short lived singlet species with a lifetime of 10^{-8} - 10^{-8} seconds. This excited state may undergo intersystem crossing which results in the excited triplet state which has a longer lifetime of around 10^{-3} seconds, allowing it to participate in chemical reaction with other molecular species (Knox and Dodge, 1985; Malik *et al.*, 1990)

Excited state porphyrins may cause cellular damage via two possible mechanisms; either a type I or a type II reaction. The definition of these two types has recently been clarified, and is dependent on the primary interaction of the activated molecule (Foote, 1991). A type I reaction is one where the activated molecule reacts with either the substrate or the solvent. A type II reaction is one where the activated molecule reacts with oxygen. The type I reaction results in the generation of either radicals or radical ions by either hydrogen atom or electron transfer. The type II reaction occurs mainly via energy transfer to produce singlet oxygen. However, electron transfer to produce the superoxide ion, which is often classed as a type I reaction, would, under this definition be classed as type II. (Canistraro and Van De Vorst, 1977; Buettner and Oberley, 1980; Grossweiner *et al.*, 1982; Davila and Harriman, 1988; 1982; Moan, 1988; Van Steveninck *et al.*, 1988; Hooper and Phinney, 1988(a); Malik *et al.*, 1990;). The cellular damage caused by photoactivation of porphyrin molecules, by visible light, proceeds primarily via the generation of the singlet oxygen molecule (Stenstrom *et al.*, 1980; Felix *et al.*, 1983; Kessel,

1984; Malik, 1990;).

The species generated by the irradiation of protoporphyrin IX have been studied (Cox et al., 1979; Cox and Whitten, 1982; Horsey and Whitten, 1978; Krasnovsky, 1979). Cox et al. (1979) showed that irradiation of protoporphyrin IX may result in the production of the superoxide anion via the type I electron transfer mechanism. More recently Cox and Whitten (1982) studied the various species of molecule which accumulate after irradiation of protoporphyrin IX in the presence of molecular oxygen. Virtually all products derive from singlet oxygen attack. This illustrates that the singlet oxygen molecule is the primary and predominant product of the photoactivation of protoporphyrin IX under the particular conditions studied. This confirms the studies of Krasnovsky (1979) which showed that on irradiation of protoporphyrin IX in the presence of molecular oxygen, generation of singlet oxygen was more efficient than the generation of superoxide radical.

Cox and Whitten (1982) investigated the reaction of protoporphyrin IX with the singlet oxygen it generates; their results showed this reaction had a low rate constant compared with other porphyrins; being lower than bacteriochlorophyll and other chlorophyll derivatives. This suggests an increased likelihood that singlet oxygen generated in vivo will escape quenching by the protoporphyrin IX molecule, allowing interaction with other cellular species.

Of particular interest is the observation that a number of photobleaching herbicides, such as oxadiazon, exhibit their effect by perturbing cellular metabolism, leading to the accumulation of Protoporphyrin IX. The porphyrin thus accumulated renders the plant sensitive to photoinactivation (Becerril and Duke, 1989; Nicolaus et al.,

1988; Sandmann and Boger, 1988).

Moan (1986) suggested that free radicals probably play an insignificant role in biological systems. However, they are undoubtedly of significance in the absence of molecular oxygen, and are likely to play an accessory role to damage elicited by singlet oxygen. The competition between type I and type II sensitization of liposomes has been studied (Grossweiner *et al.*, 1982; Rossi *et al.*, 1981; Scofienza *et al.*, 1980). The predominant reaction mechanism was found to be dependent on a number of parameters including aggregation state of the photosensitizer, its adhesion to the target and the ionic environment. Free radicals have been shown to have a primary role in some photoactivations (Hooper and Phinney, 1988a; Felix *et al.*, 1983). Grossweiner *et al.* (1982) showed anaerobic type I sensitization of liposomes by hematoporphyrin. In addition anaerobic bacteria may be sensitized in the absence of molecular oxygen (Malik, 1990). Under these conditions free radicals would be the primary photoreactive species.

The photoactivation of porphyrin molecules has a number of potential effects on cellular systems. The membranes are a major target for photodynamic processes leading to impaired transport and permeability (Kessel, 1984). Membrane lipids may become peroxidised. This is an autocatalytic process in which the lipid undergoes an oxidation to form a carbon centred radical. This radical may be propagated by interaction with other lipid molecules or oxygen. Termination occurs when two radicals interact to form nonradical products (Burton and Ingold, 1984). The peroxidation reaction generally results in increased membrane permeability, alteration in membrane fluidity, and the loss of activity in some enzymes eg. cytochrome P450. Lipid peroxidation may be initiated by a number of

means including porphyrin irradiation, and has been studied in a number of systems. In many of these emphasis has centred around oxygen and the singlet oxygen molecule in the initiation of the peroxidation reaction (Anderson and Krinsky, 1973; Lamola et al., 1973; Pooler, 1989; Roshchupkin et al., 1975). Of particular interest is the study of erythropoietic protoporphyria a genetic disease in which red blood cells accumulate protoporphyrin (Hsu et al., 1971; Lamola et al., 1973). Sufferers from the disease exhibit cutaneous photosensitivity. Red blood cells containing excess protoporphyrin are particularly sensitive to light at 400nm, the photohemolysis requires oxygen and is accompanied by lipid peroxidation which appears to be initiated by singlet oxygen.

Irradiation may result in the oxidation of sulphhydryl groups which have been shown to be important in the maintenance of membrane integrity (Edwards et al., 1984). Because of the reactivity of some amino acids, such as histidine, membrane damage has been suggested to proceed mainly via the photodamage of proteins (Mattheson et al., 1975). These proteins may undergo cross-linking or photooxidation, which alters their structure and activity.

Protein damage may result in ion pumps being disrupted which inhibits the transport of essential metabolites. Damage to cytoplasmic membranes may result in an enhanced uptake of porphyrin into the cell while attacks on lysosomal membranes may result in the leakage of harmful lysosomal enzymes into the cytoplasm. The result appears to be an influx of water, and a decrease in the energy state of the cell, leading to a loss of viability (Bertoloni et al., 1989; Fiel et al., 1981; Khanum and Jain, 1989; Kessel, 1988; Malik, 1990; Moan, 1988; le Nouen, 1989; Praseuth et al., 1988).

Factors that affect these processes have been investigated. Oxygen tension was found to be the limiting factor in hematoporphyrin induced photosensitization of tumour mitochondrial cytochrome c (Gibson and Hill, 1985). For other porphyrin photosensitizations requiring oxygen, see Mitchell et al., 1985; Moan and Sommer, 1985; and See et al., 1984. In a study of leukemia L1210 cells sensitized with hematoporphyrin, the time of exposure to light not only altered the site of photosensitization but also the nature of the photosensitizer (Kessel, 1988).

The structure of the porphyrin plays an important role. In general, a combination of high susceptibility to photodegradation, and enzymatic degradation will result in a photochemically inefficient photosensitizer (Kimmel et al., 1989). In addition, binding of the porphyrin to, or entry into, the membrane appears to be the most likely prerequisite to the cytotoxic response to photoirradiation (Ehrenberg et al., 1985; Gibson, 1987).

Aggregation of porphyrin greatly reduces its capacity to generate singlet oxygen (Lambert et al., 1988; Moan, 1988). The more hydrophobic the molecule the more likely it is to aggregate in the predominantly hydrated cell. The cytoplasmic membrane has been identified as an important site in porphyrin photosensitization. To interact effectively with the predominantly hydrophobic membrane, the porphyrin molecule must be predominantly hydrophobic (Kessel, 1977). These two observations suggest that the most effective photosensitizing porphyrin would be at neither extreme of hydrophobicity or hydrophilicity. This may be of little significance to intracellular porphyrins which may accumulate gradually and insert into the membrane before aggregation becomes significant.

The structure of the target cell, in particular its membranes (Bartoloni et al., 1984; Mathews, 1980) affects the degree of

photosensitization. Gram positive bacteria are photo-sensitive to a number of porphyrins, whereas the outer membrane of Gram negative bacteria makes them resistant (Dahl et al., 1989(b)). However once cells are permeabilized allowing the uptake of porphyrin, or the penetration of toxic oxygen species, both show similar sensitivity. Casey and Parks (1989) used an auxotrophic mutant of Saccharomyces cerevisiae, supplemented with different sterols, to examine the effect of sterol content on hematoporphyrin sensitized lipid peroxidation. It was suggested that peroxidation occurred by the addition of singlet oxygen to double bonds, and subsequently proven that supplementation with stanols, which are completely saturated, rendered the yeast resistant to photolysis.

Photoactivated porphyrins have been implicated in both carcinogenic and mutagenic effects. Porphyrin photoactivation may lead to the damage of nucleic acids, in particular DNA. Praseuth et al. (1986) showed that anionic porphyrins are less efficient than cationic porphyrins at sensitizing DNA. This probably reflects the affinity of cationic porphyrins for negatively charged DNA. In attacking nucleic acids, guanine residues are the predominant target for porphyrin induced strand breaks. Le Nouen et al. (1989) suggested that a type I reaction could be one of the primary processes in DNA photosensitization.

1.C. Carotenoids.

1.C.1. Description of carotenoids.

Carotenoids undoubtedly form the most ubiquitous group of pigments in nature, being found in: all photosynthetic organisms, plants bacteria and algae; in some fungi; and some aerobic non-photosynthetic bacteria.

Since the yellow carotene pigment was first isolated by Wachenroder in 1831 (for ref. see Goodwin, 1980) some 500 closely related pigments have been identified. Our knowledge of their structure, function, biosynthesis, and biochemistry, has increased steadily and numerous texts have been published on the subject; (Britton, 1985; Goodwin, 1980; Goodwin and Britton, 1988).

The definition of a compound as a carotenoid derives from its chemical origin. The basic precursor being the acyclic $C_{40}H_{56}$ polyene, lycopene. The pathway of synthesis proceeds from mevalonic acid which is converted into isopentyl diphosphate, which may in turn be isomerized and forms the basic C_5 unit from which the long chain is synthesized (Llaen-Jenssen, 1985). Prenyl transfer between C_5 units produces successively, chains of length C_{10} , C_{15} , and C_{20} (geranyl geranyl diphosphate (GGDP)). The C_{40} molecule phytoene is formed from two molecules of GGDP (Goodwin, 1988). Phytoene undergoes stepwise dehydrogenation through a series of intermediates to form lycopene (Porter and Lincoln, 1950).

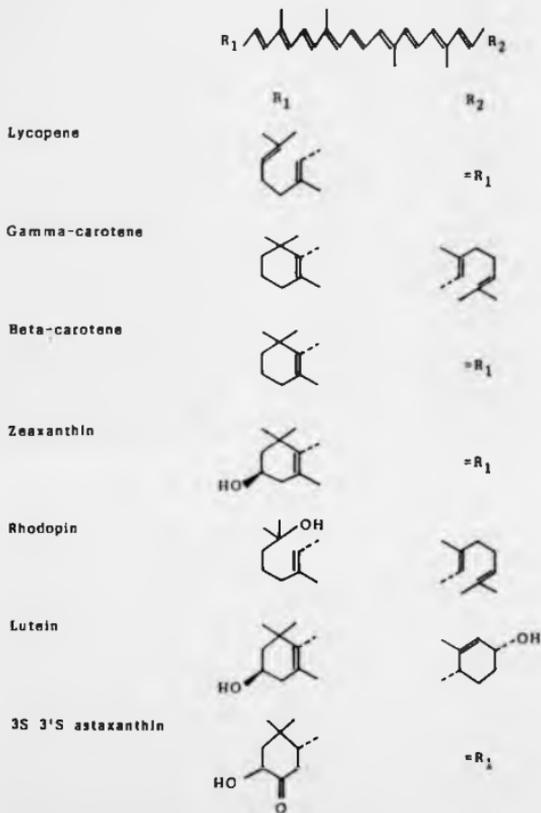
It is believed that carotenoid biosynthesis is driven by a multi-enzyme complex closely linked to, or integral with the plasma membrane (Aragon et al., 1978; Cerda-Olmeda and Torres-Martinez, 1979). In a study of the carotenogenic enzymes of Narcissus pseudonarcissus L., Kreuz et al. (1982) showed these enzymes were found associated the chromoplast membranes: some enzymes were peripheral; whilst others were integral. The large variety of different carotenoids seen are formed from chemical alteration of the precursor lycopene which may proceed by:- a) hydrogenation b) dehydrogenation c) cyclization d) insertion of oxygen in various forms e) double bond migration f) methyl migration g) chain elongation h) chain shortening and i) glycosylation (Goodwin, 1980; Foppen, 1971).

Examples of some modifications are shown in Figure 4. Cyclization may result in monocyclic or bicyclic carotenoid such as gamma-carotene or beta-carotene respectively. Addition of oxygen may occur via keto substitution, or hydroxylation of the ring, or the long chain, egs. 3S 3'S astaxanthin, zeaxanthin, or rhodopin. Different isomeric forms result from the many chemical modifications. Attachment of the OH group to the rings of a beta-carotene precursor results in either zeaxanthin, or lutein, depending upon the chirality of the OH group about the 3' carbon. Such OH groups may become methylated, such as in spirilloxanthin.

Larger carotenoids with C₄₅ and C₅₀ backbones have been isolated from several Gram positive, non-photosynthetic bacteria, these are produced by the addition of C₅ units to a C₄₀ precursor.

The biological significance of the modifications remains to be determined. However, the reaction centres of Rb. sphaeroides show selectivity for carotenoids which contain polar functional groups eg. methoxy groups (Cogdell and Frank, 1987). As such the modifications may determine the specificity of interaction with the reaction site. Cogdell and Frank (1987) also speculated that the inherent flexibility of the polyene chain of the carotenoid molecule may contribute towards its ability to interact with various different molecules. Additionally, as will be discussed later, it has been suggested that the isomeric form of the carotenoid determines its efficiency as either a light harvesting pigment, or as a light protective pigment (Koyama et al., 1990).

Figure 4. Some carotenoids produced by the modification of a lycopene precursor: For explanation see text. Taken in part from DiMascio *et al.*, 1989; and Goodwin, 1980.



1.C.2. Techniques Used in the Study of Carotenoids.

Carotenoids may be extracted from biological samples in a number of different ways which are determined by the nature of the tissue, the types of carotenoid being isolated, and the inherent instability of carotenoids to the action of oxygen, light, heat, etc. (Britton, 1985). Individual carotenoids may be purified by a number of chromatographic techniques including column, thin-layer, and high performance liquid chromatography. By varying the ratios of solvents, separation of compounds of widely varying polarity may be achieved (Krinsky and Welankiwar, 1984; Ruddat and Will III, 1985).

Once isolated the complex features of the carotenoid molecule may be determined by a number of techniques. Foremost of these is absorption spectroscopy. The unsaturated chain structure of carotenoids produce a characteristic triple peak. The position, intensity, and fine structure of individual peaks is affected by a number of factors, eg: a) the degree of conjugation in the system; b) the influence of substituted chemical groups, and c) the isomeric form of the molecule (Goodwin, 1980). A large number of diagnostic tables exist listing carotenoid absorption maxima in commonly used solvents (Foppen, 1971; Britton, 1985). Absorption spectra are diagnostic for many carotenoids and may be supplemented by other spectroscopic techniques, namely: infra-red, nuclear magnetic resonance, and mass spectroscopy. Electron spin resonance, and raman spectroscopy have been used to investigate the role of singlet and triplet states in the reactions of carotenoids. X-ray crystallography of reaction centres isolated from Rh sphaeroides has been used to study the spatial arrangement and possible points of interaction of the carotenoid molecule within the

reaction centre (Ducruix and Reiss-Husson, 1987; Frank et al., 1987).

The structure of the carotenoid molecule may be investigated in the study of its chemistry. For instance isomerization of 5,8-epoxides by hydrochloric acid causes a shortening of the chromophore which is seen as a marked hypsochromic shift in the molecules absorption spectrum.

The biochemistry of carotenogenesis has been studied by a wide variety of techniques. Synthetic pathways may be examined by the use of radioactive precursors of MVA. or IDP. allowing subsequent isolation of radioactive intermediates. Carotenoids have been synthesized in vitro in order to isolate intermediates for physical analysis. To study the specific interactions of carotenoids with other species, carotenoids have been synthesised which are linked to different molecules of interest eg. porphyrins (Moore et al., 1980). Inhibitors have proved a useful tool in both physiological and biochemical studies, for instance, nicotine prevents the cyclization reaction and causes the accumulation of the long chain molecules, usually lycopene, thus identifying the precursor for cyclization (Goodwin, 1980; Goodwin, 1986). Genetic analysis of cells mutated in carotenoid genes has proved a valuable tool. For instance the study of heterokaryons of Phycomyces blakesleeanus produced from crosses between various colour-mutants, has helped establish the sequence of the carotenoid biosynthetic pathway (Cerdá-Olmeda and Torres-Martínez, 1979).

1.C.3. The Functions of Carotenoids in Biological Systems.

Carotenoids have been implicated in a wide variety of biological phenomena. They have been shown to be possible photoreceptors in phototaxis, and a number of developmental and biochemical processes in

plants (Thomas, 1950; Schrott, 1985). They may serve as precursors for biochemical reactions (Tau 1988). They may also function in stabilizing biological membranes (Huang and Haug, 1974), as accessory light harvesting pigments in photosynthesis, and as quenchers of potentially harmful toxic oxygen species and radicals (Cogdell, 1985; Cogdell and Frank, 1987; Koka and Song, 1976; Liaasen-Jensen, 1978; Schrott, 1985; Siefertmann-Harms, 1987).

It is their roles as accessory pigments and photoprotective quenchers that seem most biologically significant. Carotenoids exhibit a large energy gap between the first excited singlet state, and the first excited triplet state (Cogdell, 1985), thus indicating their suitability for this dual role. The high singlet state being important in light harvesting, the low triplet state, in photoprotection. The suitability of carotenoids led to their study as an auxiliary photoreceptor in an artificial porphyrin based photoreceptor cell (Moore et al., 1980).

It is important to note that carotenoids only act as auxiliary pigments, since photosynthesis cannot take place in the absence of chlorophyll, and there being no evidence of a role for carotenoids in primary photochemistry (Fuller and Anderson, 1958). The ability of carotenoids to transfer captured light energy to chlorophyll was first shown by Engelman in 1884. Using motile aerobic bacteria as indicators of oxygen evolution, i.e. photosynthesis, he showed that excitation of photosynthetic algae with wavelengths of light at which only carotenoid absorbed resulted in photosynthetic reaction.

The biological significance of accessory light capture appears to be in allowing the absorption of wavelengths that are poorly or not absorbed by chlorophyll i.e. 450-570nm. This is particularly important in aquatic

systems since water filters out long wavelengths leaving predominantly blue light at depth (Siefermann-Harms 1987).

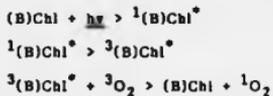
Carotenoids are found in association with the reaction centres (RC) and the light harvesting (LH) antenna of photosynthetic bacteria. The carotenoids that are present within these structures are determined by the function of primary importance required by the structure. For instance in the LH complex carotenoids with high extraction coefficients are found to fulfill the primary role of light harvesting. In the RC carotenoids which are efficient dissipators of activation energy are found to fulfill the primary role of light protection. It should be stressed that secondary roles of photoprotection of light harvesting are also fulfilled by the above carotenoids respectively (Koyama *et al.*, 1990).

The efficiency of light energy transfer from carotenoids to bacteriochlorophyll has been studied in a number of photosynthetic bacteria. Efficiency may vary in different species between 30%-100% (Duyens, 1951; Cogdell, 1985). The transfer of excitation energy from the carotenoid molecule to (bacterio)chlorophyll proceeds via a singlet-singlet transfer (Cogdell and Frank, 1987; Naqui, 1980). In the absence of energy transfer the de-excitation lifetime of the excited state carotenoid is less than one picosecond (Dallinger *et al.*, 1981). As such, the mechanism of transfer of activation energy from the carotenoid to the (bacterio)chlorophyll molecule is unlikely to proceed via the long range dipole-dipole (Forster) mechanism. The mechanism is more likely to be the edge to edge electron exchange (Dexter) mechanism which is facilitated by overlapping electronic orbitals. This illustrates the need for a highly ordered reaction centre, with the carotenoid molecules in close proximity to the (bacterio)chlorophyll (Siefermann-Harms 1987). Boucher *et al.*

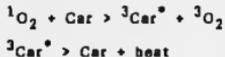
(1977) examined the effect of reconstituting reaction centres and carotenoids of Rhodospirillum rubrum. It was shown that there appear to be definite carotenoid attachment sites, and that attachment may alter the spectroscopic properties of both the carotenoid and the bacteriochlorophyll. For example, carotenoids attached to the reaction centre of Rb. sphaeroides undergo a red shift of 20nm (Cogdell, 1987). The close proximity of carotenoid to (bacterio)chlorophyll is also important to the carotenoids photoprotective function.

Many methods of photoprotection have been noted (Demmig-Adams et al., 1989). These include, mechanical processes such as leaf movements and increased reflection of light; and biochemical processes involving enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and a variety of anti-oxidants such as vitamins A and E (Carboneau et al., 1989; Thomas and Girotti, 1989). However, carotenoids play an essential part in photoprotection which gives them a major role in non-photosynthetic organisms.

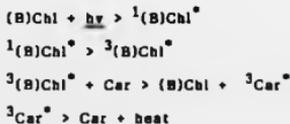
The protective role of carotenoids was illustrated in the mid-fifties (Griffiths et al., 1955; Siström et al., 1956). A carotenoid-less strain of Rhodospseudomonas sphaeroides which grew identically to wild-type in light and nitrogen was killed in the presence of light and air, i.e. oxygen. This result has been confirmed in plants using the herbicide SAN9789 which inhibits the synthesis of coloured carotenoids (Frosch et al., 1979). Upon excitation (bacterio)chlorophyll molecules are promoted to an excited singlet state ($^1(B)Chl^*$). A proportion of these molecules undergo intersystem crossing to form the long lived triplet state ($^3(B)Chl^*$). In the presence of oxygen (3O_2) the triplet state may form the potentially lethal singlet oxygen species (1O_2). Illustrated below:



Chlorophyll/carotenoid interactions have been reproduced in vitro. Boucher (1977) showed that addition of indigenous carotenoids to isolated reaction centres of Rhodospirillum rubrum confers protection against photodynamic bleaching of bacteriochlorophyll. There are two possible mechanisms by which carotenoids may protect (bacterio)chlorophyll in vivo from the harmful photodynamic effects of photoactivated pigment. The first is a quenching mechanism in which the carotenoid removes the excitation energy from the singlet oxygen molecule as shown below:



The second is a trapping mechanism in which the carotenoid molecule removes the excitation energy from the triplet state (bacterio)chlorophyll molecule as shown below:



Of the two mechanism it appears that the latter is of greater biological significance, for two reasons: 1) the quenching mechanism is diffusion

limited; 2) The extreme sensitivity of the (bacterio)chlorophyll molecule to the singlet oxygen molecule it generates ensures an essential role for the trapping mechanism in preventing its synthesis.

The mechanism by which carotenoids dissipate activation energy is, as was alluded to above, thought to be via non-radiative transmission such as heat. Demmig-Adams *et al.*, (1988) showed that the presence of carotenoids increases the amount of non-radiative energy consumption by the leaves of Nerium oleander. Ashikawa *et al.* (1988) showed that irradiation of carotenoids in the reaction centres of Rb. rubrum results in the isomerization of the predominantly 15-cis carotenoid. The most efficient carotenoids show the ability to convert to an all-trans isomer (Koyama *et al.*, 1990). This isomerisation is believed to aid the dissipation of triplet energy by converting it into kinetic energy and "then into energies of other degrees of freedom" (Koyama *et al.*, 1990).

The two key elements involved in photodynamic damage against which carotenoids act are oxygen and light.

An example of oxygen tolerance imparted by carotenoids was reported by Hartmann and Hurek, (1988). A carotenoid overproducing strain of Azospirillum brasilense showed an increased tolerance of nitrogen fixation to high ppO₂ when compared with a wild-type strain which has less pigmentation.

The broad types of light, against which carotenoids may protect, have been reported. Ultraviolet light has been shown to generate oxygen dependent lipid peroxidation (Roshchupkin *et al.*, 1975). Carotenoids may protect against such reactions and have been shown to protect against photodynamic damage to liposomes (Anderson and Krinsky, 1973). The ability of beta carotene to quench singlet oxygen (Foote and Denny, 1988)

undoubtedly allow it to quench the species required for initiation of peroxidation, however, it has also been shown to act as a lipid antioxidant scavenging lipid radicals at low physiological ppO_2 (Burton and Ingold, 1984).

Blanc et al., (1978) studied the effects of visible light, long wave UV (LWUV) (reported as blacklight), and shortwave UV (SWUV) on albino, and carotenoid producing wild-type strains of Neurospora crassa. Results showed that carotenoids could protect conidia from visible, and LWUV light, but not from SWUV. This apparent anomaly is indicative of the sites at which LWUV and SWUV have their predominant lethal action. LWUV acts predominantly at the membrane SWUV predominantly on nucleic acid (Kelland et al., 1983). This phenomena is further illustrated by the use of photosensitizing dyes. In studies by Matthews-Roth (1987) a wild-type and a colourless mutant of Sarcina lutea were used in combination with two photosensitizing dyes. Carotenoids were shown to protect against the action of toluidine blue and light, which acts primarily at the cytoplasmic membrane. However, carotenoids could not protect against acridine orange and light, which acts at both the cytoplasmic membrane and on nucleic acid. Further analysis of membrane proteins showed, despite the lethality of acridine orange, carotenoids were still protecting membrane proteins, and that lethality was due to nucleic acid damage. This protection has been reproduced in heterologous systems. Tuveson et al. (1988) showed that carotenoid genes cloned from Erwinia herbicola into E. coli could protect the heterologous host from near UV (LWUV), and the effects of toluidine blue plus light. Again protection against far UV, and acridine orange plus light was not achieved.

Carotenoids are able to quench oxidative free radicals (Burton and

Ingold, 1984). Carboneau (1983) studied the quenching of hydroxyl radicals by the carotenoids of Deinococcus radiodurans. The lethal effect of hydrogen peroxide was reduced by the addition of an iron chelator. This suggests that the effect of hydrogen peroxide, and consequently protection by carotenoid, may act via the hydroxyl radical as iron or a similar metal ion was required for the production of the hydroxyl radical from hydrogen peroxide.

Carotenoids are one of the most efficient quenchers of singlet oxygen (DiMascio et al., 1983; Foote and Denny, 1968). Foote and Denny (1968) showed that beta-carotene is capable of quenching singlet oxygen directly. Quenching of the triplet state photosensitizer or shadowing of the sensitizer were excluded in experimental controls. Their experiments used low concentrations of beta-carotene and showed no appreciable consumption of carotenoid, in some cases one molecule of beta-carotene would quench up to 250 molecules of singlet oxygen.

DiMascio et al. (1983) compared the quenching efficiency of several carotenoids. They showed that carotenoids such as retinoic acid, which have less than 7 conjugated double bonds, were unable to quench singlet oxygen. This confirms the suggestion that for a carotenoid to have a triplet state whose energy is below that of singlet oxygen (94 kJ mole^{-1}) to allow singlet/triplet transfer, the carotenoid must have more than 7/8 conjugated double bonds. This was confirmed in vivo in a study of a mutant of Sarcina lutea, whose major carotenoid contained 8 conjugated double bonds (Mathews-Roth et al., 1974). This pigment conferred no photoprotection on the organism (Mathews-Roth and Krinsky, 1970).

It was stated by Edwards et al. (1984) that "the ability of bacteria to survive irradiation is dependent on the fluidity of the plasma

membrane". The molecular mechanism by which such protection is determined is not known. However, this may illustrate one more potential facet in the protective capability of carotenoids, in that it has been reported that the carotenoid content of a membrane affects its fluidity (Huang and Haug, 1974), and as such may be important in maintaining its structure.

1.C.4. The Mechanism of Induction of Carotenogenesis.

Carotenogenesis has been shown to be light regulated in a number of different organisms; plants, fungi, photosynthetic and non-photosynthetic bacteria (Batra and Rilling, 1984; Demmig-Adams et al., 1987; Harding and Shropshire Jr., 1980; Rau and Mitzka-Schnabel, 1985; Rilling, 1984; Zalokar, 1954). Most algae synthesise their full complement of carotenoids in the dark (Rau, 1985), indicating that in this case no light induction is required.

In non-photosynthetic organisms the induction of carotenogenesis can be considered in 3 stages (Batra and Rilling, 1984; Harding and Shropshire Jr, 1980). The first being the temperature-insensitive capture of a light signal. This is followed by the light-independent, temperature-dependent synthesis of biosynthetic enzymes. In the third stage the biosynthetic enzymes directs the synthesis of carotenoid molecules, this stage does not involve protein synthesis.

The photoinduction of carotenogenesis is in many ways similar to the harmful photodynamic effects light has on some systems, except the quantity of light required for the latter is several orders of magnitude higher than that required for the stimulation of carotenogenesis (Rilling, 1982; Wright and Rilling, 1983).

In the study of carotenogenesis the identification of the photoreceptive molecule is of primary importance. The traditional approach to identify the photoreceptor for a given response is to produce an action spectrum for that response (Harding and Shropshire Jr., 1980). This technique allows some distinction between possible photoreceptors to be made. Of the spectra which show absorption predominantly in the blue region, only porphyrin molecules show absorption above 540nm. The distinction between carotenoids and flavins which absorb below this value is contentious. Both have been proposed as possible receptors in the so-called cryptochrome responses. In part the two may be distinguished in that carotenoids show a lesser absorption in the near-UV region than do flavins. Historically flavins have been favoured as receptors above carotenoids on comparisons of the photochemistry of the two types of molecules (Song, 1981). It has been argued that the short lifetime of the activated carotenoid species would make it unable to initiate photoreponses. However the interaction of carotenoids with the reaction centres of photosynthetic bacteria mentioned above, and the isolation of light induced isomers of carotenoids (Ashikawa *et al.*, 1988), make re-examination of this phenomena necessary. Examples of absorption spectra of a porphyrin; chlorophyll, a flavin; riboflavin, and a carotenoid; beta-carotene, are shown in Figure 5.

The plant photoreceptor phytochrome has been shown to control carotenoid accumulation in tomatoes (Thomas and Jen, 1975) and in developing seeds and chloroplasts (Harding and Shropshire Jr., 1980; Lichtenthaler, 1975; Schnarrenberger and Mohr, 1970). In addition a phytochrome-like photoreceptor has been identified as controlling carotenoid synthesis in the fungus Verticillium agaricinum (Valadon *et al.*,

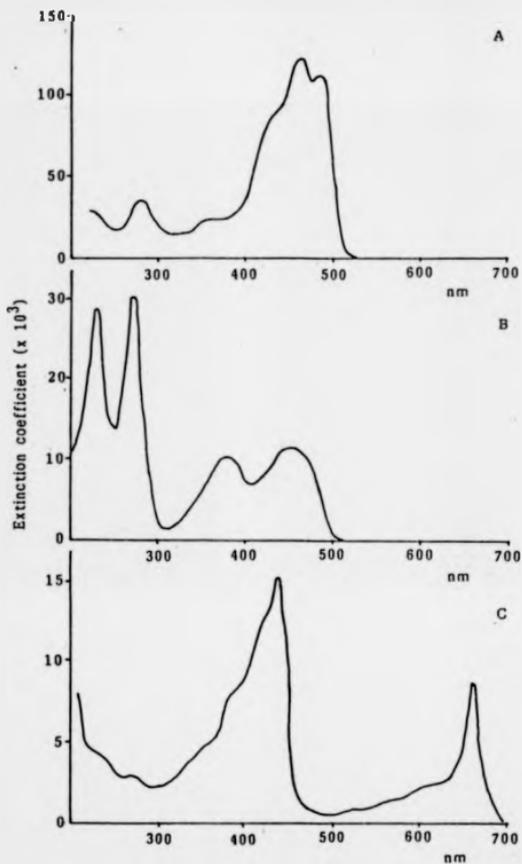


Figure 5. Examples of typical absorption spectra of some possible photoreceptors in biological systems: A) beta-carotene, B) riboflavin, C) chlorophyll. Solvents are ethanol for A, and C, and water for B). Taken from Song and Walker (1981).

1978). Two other photoreceptors to note at this point are flavins and chlorophylls. A flavin has been isolated and proposed as the cryptochrome receptor in Avens coleoptiles (Ghisla et al., 1984), and protochlorophyll(ide) has been implicated in the light induced changes in chloroplast differentiation in Euglena (Egan et al., 1975).

Identification of the photoreceptor pigment may be examined chemically; for instance, addition of hydrosulphite, which reduces flavins, will abolish flavin driven responses without affecting those driven by porphyrins. The effect of carotenoids on photophenomena may be examined by the use of mutant, or chemically induced, deficiency in carotenoid pigments.

The kinetics of carotenogenesis have been studied in a number of different organisms (Harding and Shropshire Jr, 1980; Rau and Mitzka-Schnabel, 1985). In some organisms, subsequent to the light stimulus there is a lag phase which ranges from 1 to 4 hours. This phase may be sensitive to chloramphenicol in prokaryotes, and cycloheximide in eukaryotes illustrating a requirement for protein synthesis to proceed to the next stage of carotenogenesis (Harding and Shropshire Jr, 1980). In others eg. Euglena gracilis there is no such lag (Rau, 1985). The conversion of precursor carotenoids to the end products of beta-carotene and xanthophyll occurs almost immediately post-illumination. This difference reflects the different possible modes by which light may control carotenogenesis: transcriptional, translational or post-translational.

The effects of light and oxygen on carotenoid synthesis have been studied in the photosynthetic bacteria, mainly in Rhodobacter capsulatus, Rhodobacter sphaeroides, and Rhodospirillum rubrum. Both light and oxygen exert effects on the intracytoplasmic membranes which house the

photosynthetic apparatus, on the proteins which form the light harvesting complexes, on the enzymes involved in the Calvin cycle, and on the pigments themselves. Under anaerobic conditions the cells develop an extensive intracellular membrane that comprises the photosynthetic apparatus. The amount and the composition of the membrane are regulated by the intensity of incident radiation. Decreasing light intensity leads to a compensatory increase in the photosynthetic capability of the cell. On exposure to oxygen photosynthetic capability decreases. Schumacher and Drews (1979) showed that the formation of photosynthetic apparatus follows similar kinetics whether synthesis is induced by lowering oxygen tension in the dark or by lowering light intensity under anaerobic conditions. The two stimuli, however, have been shown to act via different mechanisms (Arnheim and Oelze, 1983).

In Rb. sphaeroides it has been shown that oxygen but not light regulates 5-aminolevullinate synthase, a key enzyme in the bacteriochlorophyll synthesis pathway (Oelze and Arnheim, 1983). Biel and Marrs (1983) used promoter fusions to show that a number of the genes of the bacteriochlorophyll synthesis pathway could be regulated in response to oxygen. The effects of light and oxygen on the mRNAs encoded by harvesting proteins reaction centre polypeptides, and pigment genes have been studied. Bacteriochlorophyll, light harvesting proteins and reaction centre polypeptides seem to show coordinate expression, and differs from carotenoid expression. The mRNA for the bacteriochlorophyll genes studied by Zhu and Hearst (1988) on increasing light intensity showed a small decrease, the mRNAs for carotenoid genes showed an increase. In the presence of oxygen levels of all BChl, RC, and LH mRNAs decreased. It is thought that oxygen inhibits transcription and in some cases promotes

degradation of some mRNAs (Zhu et al., 1988).

A rapid change in the carotenoid composition of R. capsulatus occurs on transfer from anaerobic to aerobic conditions. There is a previously noted conversion of the major pigment under anaerobic conditions, spheroidene, to spheroidenone (Shneour 1982 (a) and (b)). One carotenoid gene, crtA has been shown to be activated by oxygen. This gene probably codes for an oxygenase which converts spheroidene to spheroidenone (Zhu et al., 1988). The mechanisms by which light and oxygen cause the widespread changes seen have not yet been elucidated. However, a major step forward in the study of carotenogenesis in R. capsulatus has been achieved by the cloning, sequencing and mutagenesis of a large number of carotenoid structural genes and regulatory loci which form a large gene cluster of around 11kb in size (Armstrong et al., 1989; Armstrong et al., 1990).

The light regulated proteins involved in the synthesis of carotenoids have been identified in a number of organisms. Tada, (1989) showed that in the yeast Rhodotorula minuta, the pivotal light controlled enzyme is mevalonate NADP oxido-reductase which is involved in the synthesis of isoprenoid, the primary building block for carotenoid synthesis. Using the green algae Scenedesmus obliquus, Humbeck, (1990) showed the light controlled enzyme was an isomerase responsible for the conversion of cis-lycopene to all-trans-lycopene. Experiments showed that cis-lycopene accumulated in dark grown cells, and that a mutant unable to isomerize this molecule was unable to synthesize the more saturated carotenoids when a light signal was provided.

Previously Weeks and Garner (1987) had shown two elements of control in carotenogenesis of Flavobacterium dehydrogenans. They showed

that light was essential for some pre-phytoene step and that the carotenoids produced subsequent to this depended on exogenous metabolites eg. thiamine, and on the level of aeration. Aeration, nutrient supplements, temperature and pH have been studied in a number of other systems. Some of these effects may be interpreted simply as a reflection of the increased metabolic capability of the cell (Friend and Goodwin, 1954), however most do not fit this category.

Using a Mycobacterium species, Rilling (1964) showed that oxygen was participating directly in the photoinduction of carotenogenesis, and not just as an electron-acceptor in the electron-transport chain. MacKinney (1952) showed that temperature variation resulted in both quantitative and qualitative differences in the carotenoid content of Rhodoturia glutinis. The synthesis of beta-carotene in Phycomyces blakesleeanus is stimulated by supplementation with vitamin A, and by mating; the latter effect is mediated by the production of trisporic acids. Supplementation with beta-carotene inhibits carotenogenesis indicating negative feedback is occurring (Cerdeira-Olmeda and Torres-Martinez, 1979). Variation in pH may effect carotenogenesis by maintaining or dissociating the photoreceptor/metal ion complex required for capture of the light signal (Batra and Rilling, 1964).

Control of gene expression by metalloproteins has been shown in a number of organisms (Hooper, 1988(b)). Several proteins that interact with DNA contain zinc ions, but of particular relevance to photoinductive processes, some metalloproteins are sensitive to oxidation by molecular oxygen or by photo-induced radicals. For instance photooxidation of Mn^{2+} produces Mn^{3+} which is a strong oxidant and could destroy its binding site on the protein moiety. Hooper and Phinney (1988) showed that light induction of the P21 coat protein of Arthrobacter spp. despite being a

photooxidative effect, did not involve singlet oxygen, or hydrogen peroxide. They discovered that de-repression of the gene for P21 involved the dissociation of a metal ion, probably Mn^{2+} , from a repressor protein by a photooxidative response, which may be mimicked by a decrease in pH.

It is immediately apparent why a developing seedling requires light control over the synthesis of carotenoid pigments. The etiolated seedling does not require carotenoid pigment in the dark conditions under which it develops, therefore to prevent the synthesis of such is a means of conserving the seeds limited nutrient supply. However, it is also of particular importance to a photosynthetically active plant that the concentration of carotenoid is tightly regulated, since competition with chlorophyll for excitation energy may result (Rau, 1982). A reversible method of control is illustrated in the xanthophyll cycles seen in plants and some classes of algae. A xanthophyll cycle is characteristically one in which there is a stoichiometric shift in pigment content under varying conditions of illumination; no net change in total carotenoid occurs (Yamamoto, 1985).

Light has been shown to regulate the accumulation of a xanthophyll, zeaxanthin, in the higher plant Hedera helix (Demmig-Adams et al., 1989, and therein). Conversion of the precursor molecule, violoxanthin, to zeaxanthin occurs in the thylakoid membrane. The de-epoxidase which catalyses this conversion is active only when the proton concentration on the inside of the membrane is high. As such, it is only active when more photons are absorbed, and consequently more protons accumulate, than can be utilized in photosynthesis. An additional point of regulation occurs in that the photosynthetic state also regulates the availability of the precursor violoxanthin (Yamamoto, 1979). On return to low light intensities

zeaxanthin is converted to violoxanthin by an epoxidase enzyme with an optimum pH of 7.5 i.e. low proton concentration (Demmig-Adams et al., 1989).

1.D. The genetics of carotenogenesis in M. xanthus, and the types of carotenoids produced.

1.D.1. The Carotenoids of Myxobacterales.

The types and distribution of carotenoids have been studied in several species of Myxobacterales. Published work includes Stigmatella aurantiaca, Sorangium compositum, and Myxococcus fulvus (Kleinig, 1974, Kleinig, 1975; Kleinig and Reichenbach, 1969; Kleinig and Reichenbach, 1974; Kleinig et al., 1970; Kleinig et al., 1971; Reichenbach and Kleinig, 1971). The types of carotenoid seen in M. fulvus are similar to those seen in M. xanthus (H.Reichenbach, pers. comm.).

The carotenoid content of a log phase dark grown culture of M. fulvus is approximately 0.03% of dry weight acetone extracted cells. This value rises to 0.06% in illuminated late log phase cultures. There are some 50-60 different carotenoids present of which 24 have been biochemically analysed (for list see ref. (Reichenbach and Kleinig, 1971). It appears that myxobacteria contain only acyclic and monocyclic carotenoids, however all of the Porter-Lincoln series are seen (Porter and Lincoln, 1950). Most carotenoids occur in trace amounts, however, three compounds form the majority by weight. The chemical structures of these carotenoids are shown in Figure 6. The most prevalent carotenoid is the red pigment myxobacton, which makes up around 81% of total carotenoid content. The second most prevalent is 4-keto-torulene comprising 11.3%. Myxobactin, an orange

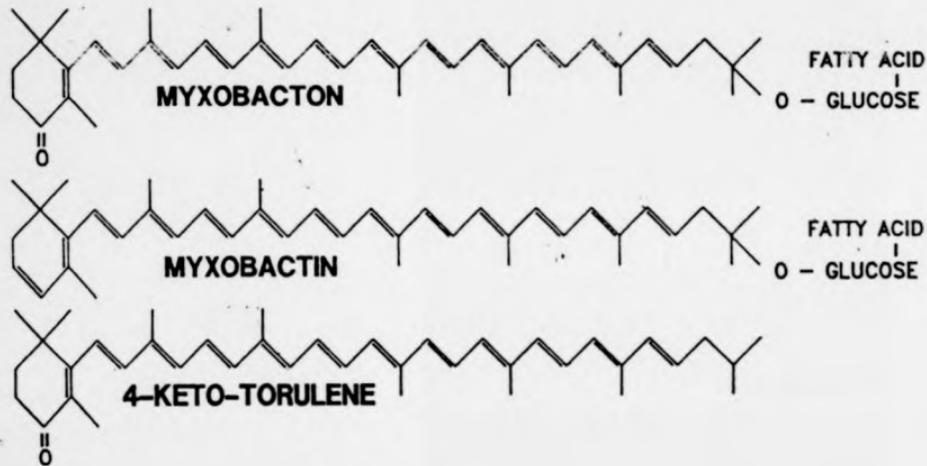


Figure 6. The major carotenoid products of *M. xanthus*: A) myxobacton; B) myxobactin; C) 4-keto torulene, (Kleing, 1974);

pigment comprises 5.9% of total carotenoid content. These values may vary depending on culture conditions (Kleinig and Reichenbach, 1973; Kleinig et al., 1970). Myxobacton and myxobactin are both tertiary glucosides, the acylated glucose moieties they contain appear to be unique to the Myxobacterales. The carotenoids appear to be very stable under in vivo conditions, with no evidence of carotenoid degrading enzymes (Kleinig and Reichenbach, 1973; Kleinig, 1975).

Studies using inhibitors of carotenoid synthesis in M. fulvus, such as nicotine, and SAN6706 have shown that carotenogenic intermediates accumulate in the cytoplasmic membrane (Kleinig, 1974; Kleinig, 1975; Kleinig and Reichenbach, 1973). This suggests the enzyme system responsible for carotenoid synthesis may be membrane associated. The nature of the enzyme system has not yet been determined.

Total specific content of carotenoid remained constant despite the effect of several inhibitors. These inhibitors caused the accumulation of different intermediates, which suggests that the structure of the molecule eg. glucoside or carotene, cyclic or acyclic, is not the determining factor in the regulation of specific content. This leaves the possibility that chromophore length may be the determining factor in carotenoid accumulation. As previously stated chromophore length i.e. degree of conjugation, is important in determining efficiency of photoprotection (Mathews et al., 1974), and therefore seems a logical criteria by which the cell may regulate carotenoid content.

1.D.2. Identification of the genetic loci involved in carotenogenesis in M. xanthus.

The genetic analysis of carotenogenesis in M. xanthus has involved a collaboration between the research groups of D.A.Hodgson and F.J.Murillo. Work at D.Hodgson's laboratory has centred around the carQRS locus. Work at F.Murillo's laboratory has centred around the carABL, carC, and carD loci. The carQRS locus contains elements that perceive the light signal and initiate the transcription of structural carotenoid genes. The carABL locus encodes a number of structural genes carB, carI and at least one other, carA encodes a regulatory element (Balsalobre et al., 1987; Martinez-Laborda et al., 1988). The carB gene encodes an enzyme that acts prior to the synthesis of phytoene in the Porter-Lincoln series (Porter and Lincoln, 1950). The carI gene is believed to code for lycopene cyclase, (F.J.Murillo, pers. comm.) The carC locus is believed to encode part of the phytoene dehydrogenase complex and probably consists of more than one gene (Martinez-Laborda et al., 1990). The carD gene encodes a protein which appears to be involved in the regulation of the carQRS region (F.J.Murillo, pers.comm.).

Random Tn5 mutagenesis led to the identification of two insertions designated omegaDK1910 and omegaDK1911 which resulted in the constitutive formation of carotenoids (Car^C). Both Tn5 insertions were found to map to identical positions (Murillo and Hodgson, unpubl. results). When this transposon mutated region was reintroduced into a wild type strain, thus creating a merodiploid, a Car^C phenotype resulted, indicating that the effect of the insertion is dominant. This was interpreted as resulting from the constitutive expression of some activator caused by the

transposon insertion. It was further implied that this activator was specifically regulated in wild type cells, and that light was the probable inducer of the activator.

Two pieces of evidence confirm the presence of an activator in the carQRS locus. Firstly deletions and insertional inactivation of the region resulted in the loss of ability to produce carotenoids. Secondly fusions of the lacZ gene to the carB and carC structural genes showed that expression from these genes was made constitutive in the presence of the transposon insertion. The carB and carC loci are not linked to the carQRS locus indicating that the latter must code for a trans activating factor

The piece of DNA containing the proposed light regulated promoter was cloned from wild type cells and placed in a promoter probe vector. This vector contains a promoter-less E.coli lacZ gene which serves as an easily assayable reporter of promoter expression. White light caused an increase in beta-galactosidase activity confirming that a promoter in this region was light inducible. Cells were exposed continuously to light of 120 microE m⁻² s⁻¹, this resulted in an increase in promoter activity to around 380 Units. The dark control produced only 3-8 Units (Hodgson, 1987). Carotenoids were seen accumulating in the culture about two hours before promoter activity peaked, after which activity decreased to a basal induced level at around 150 Units. The Tn5 insertion at omega1910/1911 was tested in the promoter probe vector and was found to be constitutively expressed in the dark.

The nature of the regulation of the light inducible promoter of the carQRS locus (termed p^{QRS}) was investigated. The effect of various Car^C mutants, on the expression of p^{QRS} as measured by lacZ activity was

determined. A number of mutants were isolated which caused constitutive expression of p^{QRS} . These mutants, termed carR mutants, mapped to the carQRS region. In addition mutants at the carA locus resulted in a Car^C phenotype in which p^{QRS} was still light inducible. This confirmed previous reports that constitutive expression of carB results from mutations in carQRS or in a linked locus carA, implicating carA as a cis acting regulator of carB (Balsalobre *et al.*, 1987).

The carR mutants which led to the deregulation of p^{QRS} were mapped and found to lie downstream of p^{QRS} . This implied that the carQRS locus encodes not only a trans activator of carotenoid structural genes, but also a negative autoregulatory element.

1.D.3. Sequence analysis of the carQRS locus and the functions of the open reading frames contained therein.

The DNA surrounding the carQRS region has been sequenced (McGowan, 1989). Sequence analysis identified three translationally coupled open reading frames (orfs) downstream of p^{QRS} termed orf I, II, and III. One orf, termed orfX transcribed divergently from orfs I, II, III. One further orf, termed orfY downstream of orfs I, II, and III and transcribed from the opposite strand to these orfs. Orfs II and X appear to be integral membrane proteins. No homology to published sequence was found for orfs I, II, III, or Y, however orfX does show homology to cytochrome oxidases. None of the orfs exhibit DNA binding motifs. The transcriptional start site of p^{QRS} has been identified using primer extension (McGowan, 1989). The -10 and -35 sequences show some similarity to the E. coli stress consensus promoter. The function of sequences upstream to the promoter are

currently being examined by deletion analysis.

The functions of the orfs have been further investigated. As mentioned previously several carR mutants were isolated which resulted in the deregulation of p^{QRS} . These mutations mapped to orfII and hence this was termed carR and encodes the negative autoregulatory element previously described. Subsequently orfI was termed carQ and orfIII, carS. The second function attributed to the carQRS region was the activation of transcription from structural carotenoid genes. Two pieces of evidence indicate that the carS gene encodes the activator. Firstly, all of carQ and most of carR were deleted from an otherwise wild type strain. The remaining gene, carS was placed under the control of the constitutive promoter of the Kanamycin resistance determinant of Tn5. This strain showed a Car^C phenotype. Secondly, insertional inactivation of carS results in a strain which is unable to synthesise carotenoids in the light or the dark (Car⁻). It was further shown that the inactivation of carS did not affect induction of p^{QRS} illustrating that it does not play a part in the activation of the carQRS region (D.Hodgson pers. comm.).

The orfX has been disrupted in vitro, in vivo this inactivation has no effect on viability or carotenogenesis, as such this orf was termed the gene of unknown function A (gufA). The function of carQ is addressed in this study, which has led to ongoing investigations into a further remarkable mechanism in the regulation of p^{QRS} .

1.D.4. The proposed mechanism of induction of carotenogenesis in M. xanthus.

When this study commenced the carQRS region had not been sequenced and an initial hypothesis as to the mechanism of induction of carotenogenesis was proposed and is shown in Figure 7 (Hodgson, 1987). It was proposed that the region now termed carQRS coded for an autoregulatory repressor which on interaction with light released the promoter for transcription resulting in the synthesis of the trans activator. This activator caused the synthesis of the structural genes. After the publication of the sequence of the carQRS region and the discovery of carD this hypothesis was modified and is shown in Figure 8. It was clear that the CarR protein could not interact with p^{QRS} itself which gave a possible role to carD. The CarR protein is located in the membrane, where it may receive a light signal transmitted via the photoreceptor molecule, proposed to be Protoporphyrin IX. This received the CarR protein signals to the p^{QRS} promoter, possibly via the CarD protein. This signal results in transcription from the carQRS operon, which leads to the activation of the structural genes for carotenogenesis. This leads to the synthesis of carotenoids which in turn feedback to reduce the light signal to the CarR protein at the membrane, and hence p^{QRS} . This proposed mechanism is however in a fluid state and is constantly evolving.

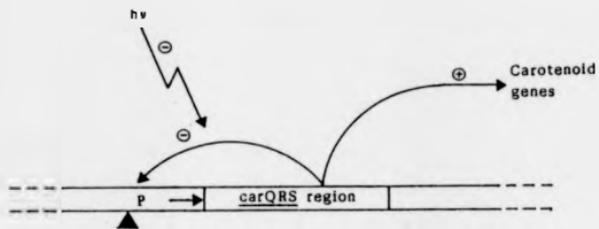


Figure 7. The original proposed mechanism for the induction of carotenogenesis in *M. xanthus*: Showing the production of an activator of carotenoid structural genes, and an autoregulatory repressor of the light inducible promoter whose action is inhibited by light. (▲) indicates the transposon insertion site omega-DK1910, and omega-DK1911. From Hodgson (1987).

Figure 2. A model for the induction of carotenogenesis in M. xanthus: The light-inducible promoter (p^{QRS}) P controls the expression of three open reading frames carO, carR and carS. carR encodes a membrane bound protein which is coupled to the product of the carQ gene. Propagation of the light signal via protoporphyrin IX in the membrane, to the CarR protein causes the release of the CarQ protein. This stimulates expression from p^{QRS} which may involve the product of the carD gene. Activation of p^{QRS} leads to the synthesis of the CarS protein which is an activator of carotenoid structural genes carB and carC, which leads to the synthesis of carotenoids. The carotenoids feedback to quench the light signal to the CarR protein, thus stimulation of expression from p^{QRS} is reduced.

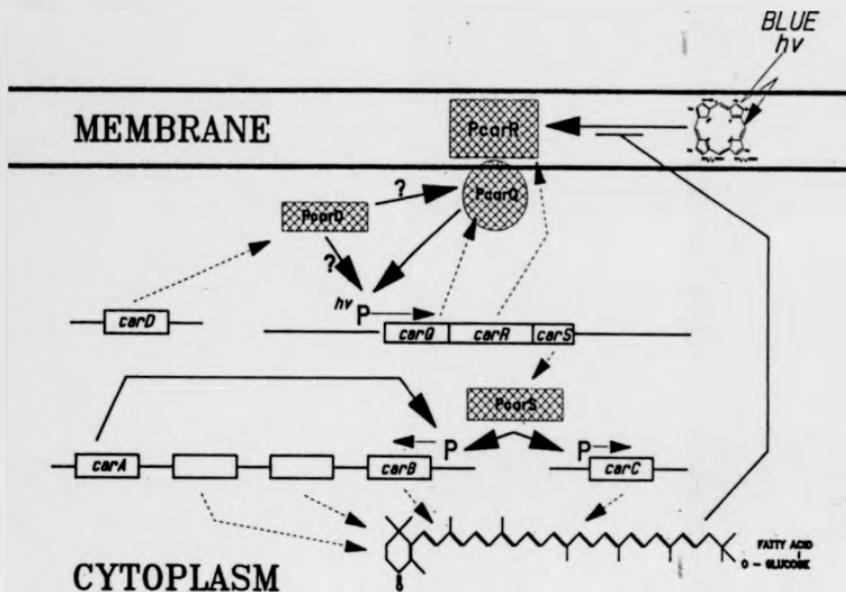


Figure 8.

MATERIALS AND METHODS.

Note: materials listed outside parentheses are detailed in the Materials section at the end of each section.

2.1. Techniques for the manipulation of nucleic acids.

2.1.1. Techniques for the manipulation of DNA in solution.

2.1.1.1. Restriction endonuclease digestion of DNA.

Conditions for optimal enzyme activity were achieved according to manufacturers instructions. DNA was digested at 37°C in typically 10-25 micro-l, for 1-2 hours. When digestion with enzymes with incompatible requirements was required the enzyme requiring the lowest salt concentration was digested to completion first, after which the salt concentration was adjusted, and the second enzyme was added. When this was not possible DNA restricted by the first enzyme was isolated from the restriction mix by phenol extraction and ethanol precipitation after which the second digest was performed.

Materials:

(10x buffers (used according to manufacturers instructions)).

2.1.1.2. Ethanol precipitation of DNA.

To facilitate precipitation, 2 micro-l of glycogen (10mg/ml) and one tenth volume of 3M sodium acetate was added to the DNA in solution. To the mixture 2.5 volumes of ethanol were added, and the mix kept at -20°C

for at least 2 hours. DNA was obtained by pelleting in a bench microfuge, the supernatant was removed and the pellet dried under vacuum before resuspending the DNA in the required volume of TE.

Materials:

Glycogen, TE., (3M sodium acetate, ethanol).

2.1.1.3. Phenol extraction of DNA.

The volume of DNA solution was increased to 100 micro-l with TE. in a 1.5ml eppendorf tube. To this was added 2 micro-l of glycogen (10mg/ml), 10 micro-l of sodium acetate (3M), and 110 micro-l of Derbyshires reagent. This was mixed thoroughly and spun in a bench microfuge for 5 minutes. The aqueous layer was decanted into a new tube, phenol residue was removed by washing the aqueous phase with several changes of ether. The DNA was then recovered by ethanol precipitation (see Methods 2.1.2.).

Materials:

Derbyshires reagent, Glycogen, and TE., (3M sodium acetate).

2.1.1.4. De-salting of DNA.

DNA was de-salted using a G-28 sephadex column. The column was prepared as follows. A hole was punctured in the bottom of a 0.5ml eppendorf tube using a 25 gauge needle. 10 micro-l of glass beads (0.17-0.18 mm in TE.) was layered on the bottom of the tube. On top of this approximately 1ml of the sephadex suspension was added, a small addition was fitted to the top of the tube to facilitate the extra volume. The

sphadex was compacted by centrifugation at 2500 rpm for 5 minutes. The extension was removed and the 0.5 ml eppendorf was placed inside a 1.5ml eppendorf. The DNA sample was added to the top of the column which was then centrifuged as above. The de-salted DNA collects in the 1.5ml eppendorf.

Materials:

Sephadex G-28, (glass beads).

2.1.1.5. Ligation of DNA restriction endonuclease products.

DNA fragments were mixed in an approximate ratio of 1:10: vector:insert. Ligations were carried out in the smallest volume possible with a minimum of 10 micro-l. To achieve this it was sometimes necessary to concentrate DNA in solution by precipitation. To the mix of DNA products was added one tenth 10x ligation buffer, and 5 Units of T4 DNA ligase. The ligation mix was incubated at 15°C O/N before transformation into a suitable competent host was performed (section 2.1.3.)

Materials:

10x ligation mixture.

2.1.1.8. Production of blunt-ends from overhanging ends.

The method of blunt-ending is dependent on the nature of the overhang generated by restriction of the DNA. If the overhang is 5' then the "klenow" fragment of DNA polymerase I is used to fill the recessed terminus. If the overhang is 3' this is digested to a blunt end using the exonuclease activity present on T4 DNA polymerase I. The method for 5'

overhangs is as follows: The restricted DNA was isolated from the restriction mix by phenol extraction (see Methods 2.1.1.3.). The DNA was incubated in routinely 10 micro-l of klenow buffer (1x) plus 1 micro-l of E. coli DNA polymerase I, klenow fragment, and incubated at room temperature for 30 minutes. The DNA for blunting of a 5' overhang is prepared in the same way. This time it is incubated in TA buffer plus 1 micro-l of T4 DNA polymerase. The mixture is incubated at 37°C for 30 minutes.

Materials:

10x Klenow buffer, 10x TA buffer.

2.1.2. Gel electrophoresis of DNA and its isolation from solid matrix.

2.1.2.1. Gel electrophoresis using agarose as the solidifying agent.

Agarose gel electrophoresis was used for visualizing and/or isolating DNA of above 1kb. 0.5% agarose gels of 1x TBE, were routinely used. The gel was buffered using 1x TBE, containing ethidium bromide at 0.5 micro-g/ml. DNA was loaded onto the gel in agarose loading buffer (1X) and developed at a constant voltage of 30-35 Volts O/N, or at 80 Volts for 4-6 hours. A long wave UV transilluminator was used to visualize the DNA. DNA was photographed under short wave UV illumination using a polaroid camera.

Materials:

TBE., agarose loading buffer, (ethidium bromide (10mg/ml)).

2.1.2.2. Gel electrophoresis using acrylamide as the solidifying agent.

Acrylamide gel electrophoresis was used for visualizing and/or isolating DNA of below 1kb. Before loading onto the gel the DNA solution was de-salted according to methods 2.1.1.4. and mixed with acrylamide loading buffer (1X). Gels (5% acrylamide, 0.13% bis-acrylamide, 1x TBE.) of 15 x 30mm and 4mm thick, were routinely developed O/N at 80 Volts. The gel was stained in 1x TBE, containing ethidium bromide at 50 microg/ml before being visualized and photographed according to section 2.1.2.1.

Materials:

TBE., acrylamide loading buffer, (ethidium bromide (10mg/ml), bis-acrylamide etc.)

2.1.2.3. Isolation of DNA from solid matrix.

The DNA of interest was cut from the gel using a scalpel blade under long wave UV-illumination. It was then recovered from the gel slice using an electroeluter (Analytical model UEA, IBI Ltd.) following the manufacturers instructions. The solution of DNA was then ethanol precipitated according to Methods 2.1.1.2.

2.1.3. Techniques for the introduction of DNA into E. coli and M. xanthus.

2.1.3.1. Production of competent E. coli.

A 5ml overnight culture of E. coli was diluted 1:100 in 50ml LB., and grown with vigorous shaking at 37°C for 2½ hours. The cultured cells, at an OD₆₆₀ of around 0.2 for recA⁺; 0.4 for recA⁻, were pelleted and the

supernatant removed. Cells were resuspended in an equal volume of magnesium chloride (0.1M) and pelleted. The supernatant was removed and cells were resuspended in calcium chloride (0.1M, Analytical grade) at half the original volume. Cells were pelleted, the supernatant removed and cells resuspended in calcium chloride (0.1M analytical grade) at 1/25 the original volume. After storage at 4°C for 2 hours cells may be used for transformation. Storage O/N increases competency; storage for longer than 24 hours decreases competency.

Materials:

LB., (0.1M magnesium chloride, 0.1M calcium chloride (Analytical grade)).

2.1.3.2. Transformation of E. coli with plasmid DNA.

200 micro-l of competent E. coli were placed in a pre-chilled 1.5ml eppendorf tube. To these cells not more than 50ng of DNA was added, mixed, and incubated on ice for 30 minutes. The cells were then heat shocked at 42°C for 2 minutes. Cells may be directly plated in top agar at this stage. If expression of the resistance gene is required before selection, then 1ml of LB. is added to the transformed cells which are incubated at 37°C for 1 hour before plating.

Materials:

LB.

2.1.3.3. Preparation of P1 bacteriophage.

A 5ml O/N culture of suitable E. coli (eg. C800) was pelleted, the supernatant removed and the cells resuspended in 1ml of P1 buffer. A P1 phage stock was diluted by several logs in P1 buffer, 0.1ml of each dilution was added to 0.1ml of the plating bacteria. After incubation of the mixture at room-temperature for 20-30 minutes the cells were seeded into an double T top lawn and grown O/N at 37°C on double T agar. After incubation phage were harvested from the plate showing the highest titre which was not confluent. The phage were harvested by overlaying the plate with 5ml of suitable buffer or LB. and incubating at 4°C O/N. The buffer was centrifuged to remove any cell debris then stored over chloroform at 4°C.

Materials:

P1 buffer, LB., Double T.

2.1.3.4. P1 packaging of plasmids for transduction into M. xanthus

An overnight culture of E. coli containing the plasmid was diluted 1/20 into 5ml LB. plus kanamycin (40 micro-g/ml). This culture was incubated with shaking at 37°C until the OD₆₆₀ was approximately 0.21. To 0.5ml of this culture in a 1.5ml eppendorf was added 5×10^8 pfu of P1c100. The mixture was incubated at room temperature for 20 minutes and then used as the inoculum for 5ml LB. containing magnesium chloride (10mM), kanamycin (20 micro-g/ml), and chloramphenicol (12.5 micro-g/ml). This was then incubated O/N with agitation at 30°C. 0.1 ml of the resulting culture was used to inoculate 5ml of LB. containing the same

additions as above, and incubated at 30°C until an OD₆₆₀ of 0.21 was achieved. The culture was then incubated at 42°C for 35 minutes, then at 37°C for 2 hours. 0.1ml of chloroform was added and the culture incubated for a further 15 minutes. Cell debris were removed by centrifugation at 8500 rpm for 10 minutes. The decanted phage stock was then stored over chloroform at 4°C.

Materials:

LB., (1M magnesium chloride, chloramphenicol (25mg/ml), kanamycin (10mg/ml))

2.1.3.5. High titre P1 packaging of plasmids for transduction into M. xanthus

The protocol for the preparation of a high titre P1 transducing phage is similar to the above. The 5ml O/N culture of phage plus cells was diluted 4ml in 200ml then cultured to an OD₆₆₀ of 0.21. Subsequent treatments were identical until the final spin, at this point the phage was not stored over chloroform but was re-centrifuged at 8K and 4°C for 15 hours. The supernatant was removed to leave a delicate pellet of phage particles. This pellet was gently resuspended in TM, at 4°C and stored over chloroform.

Materials:

LB., TM., (1M magnesium chloride, chloramphenicol (25mg/ml), kanamycin (10mg/ml))

2.1.3.8. P1 transduction of plasmids into M. xanthus.

500 micro-l of a mid-exponential culture of M. xanthus (OD₆₆₀=0.8-1.0) was added to three bijoux tubes. To each of these 100 micro-l of 50mM calcium chloride was added. To each of the three tubes a different amount of phage lysate was added: 100, 200, and 400, micro-l; the first two were made up to 400 micro-l. by the addition of TM. The tubes were incubated at R.T. for 40 minutes and used to seed 2.5ml of DCY. top agar which was used to overlay a DCY. + kanamycin (20 micro-g/ml) plate. The plates were incubated O/N at 33°C then overlaid with 3 ml DCY. top agar + kanamycin (0.8mg/ml). Plates were incubated at 33°C for a further 5 days before transductants were seen.

Materials:

DCY., TM., (50mM calcium chloride, kanamycin (10mg/ml)).

2.1.4. Isolation of nucleic acids.

2.1.4.1. Rapid "mini-preparation" of plasmid DNA from E. coli. (Boiling method).

This method is a modified version of that presented by Holmes and Quigley (1980). The E. coli strain containing the plasmid to be analysed was patched onto LB. plates containing the correct antibiotic selection. After O/N incubation approximately 2-3 cm³ were harvested and suspended in 330 micro-l of STET. To the suspension of cells 10 micro-l of lysozyme solution (33 mg/ml in STET.) was added, after mixing, the cells were incubated on ice for 30 minutes. The tubes were then placed in a post-boiling water bath for 3 minutes, and centrifuged in a bench-top microfuge

for 15 minutes. The cell debris was removed with a tooth pick. The resulting solution in the tubes was made up to 330 micro-l with STET., and an equal volume of Isopropanol was added. The tubes were stored at -20°C for around 2 hours after which the plasmid was pelleted in a bench microfuge, the supernatant was removed and the pellet dried before resuspending the plasmid in 50 micro-l of TE. Such plasmid preparations must be stored at -20°C.

Materials:

TE., STET., LB., (lysozyme (10mg/ml in STET)).

2.1.4.2. Large scale preparation of plasmid DNA from E. coli.

The E. coli strain containing the plasmid to be prepared was cultured in 5ml LB. O/N. 2ml of this was used to inoculate 500ml of LB., which was cultured at 37°C O/N. The cells were pelleted by centrifugation and resuspended in 20ml of ice-cold TES, pelleted again, and resuspended in 10ml of STE. Storing the cells at -20°C O/N, at this point side the lysis process. To the 10ml of STE/cell suspension 1ml of freshly prepared lysozyme solution was added and mixed by inversion. After 10 minutes on ice, 2.5ml of 0.5M EDTA was added and mixed by inversion. After 10 minutes on ice, 16ml of Triton lysis mix was added rapidly, and mixed thoroughly by inversion. After 20 minutes on ice the mixture was centrifuged for 35 minutes at 19000 rpm and 4°C, using a Beckman J2-21 centrifuge and JA21 rotor. The supernatant was decanted through muslin into a 100ml measuring cylinder which contained 28.5g of caesium chloride. The salt was dissolved in the solution which was then made up to 38ml by the addition of TES. The solution was transferred to an oakridge tube to

which 2ml of ethidium bromide (10mg/ml) was added. After mixing the solution was stored at 4°C for at least 1 hour. The tube was then centrifuged at 15000rpm for 15 minutes using the JA-21 rotor and the supernatant decanted through glass wool into a clear Beckman "Quickseal" centrifuge tube (39 ml). The Beckman tube was topped up with a solution of caesium chloride (71.25%) and heat sealed according to manufacturers instructions.

The caesium chloride gradient tubes were spun at 45000 rpm. for between 18-24 hours using the Beckman L8 Ultracentrifuge, and a VT150 rotor. The plasmid band was visualised under long wave UV light and removed from the gradient using a 18 gauge needle and syringe. The ethidium bromide was removed from the plasmid solution by repeated washes with salt water saturated isopropanol. The volume of the plasmid solution was made up to 12ml with water and the DNA precipitated by the addition of 24ml of ethanol at -20°C O/N. The plasmid was pelleted by centrifugation at 15000rpm for 15 minutes (JA21 rotor), the supernatant was removed and the pellet dried under vacuum before being resuspended in 500 micro-l of TE.

Materials:

TES., STE., TE., Triton lysis mix, Lysozyme solution, Salt saturated Isopropanol, (ethidium bromide (10mg/ml)).

2.1.4.3. Preparation of genomic DNA from M. xanthus.

1ml of an O/N culture of M. xanthus was used as the inoculum for 100ml of DCY., which was incubated at 33°C for around 48 hours. The cells were pelleted, the supernatant removed, and the pellet resuspended in

10ml STE, 50 micro-l of proteinase K (20mg/ml), and 2ml of EDTA (0.5M, pH 8.0) were added and mixed by inversion. Cells were lysed by the addition of 1.5ml of Na-laurylsarcosine (10%) and rapidly mixed by inversion. The lysate was incubated at 50°C O/N. Genomic DNA may be isolated by one of two possible methods. The first is a rapid method in which one tenth sodium acetate (3M) is added to the lysate followed by an equal volume of ethanol. Genomic DNA may be spooled out of solution onto a sealed pasteur pipette formed into a hook at one end. The second method involves purification of genomic DNA on a caesium gradient. To the lysate 28.5g of caesium chloride was added, and the volume was made up to 38ml with TE. To this 2ml of ethidium bromide was added and placed at 4°C for at least 1 hour. The preparation was then treated in an identical manner to the caesium chloride gradients described in section 2.1.4.2. above, except that due to the large amount of DNA obtained, the purified DNA is resuspended in at least 1ml.

Materials:

DCY., STE., TE., (EDTA. (0.5M), Proteinase K (20 mg/ml), Na-laurylsarcosine (10%), sodium acetate (3M), Ethidium bromide (10 mg/ml)).

2.1.5. DNA immobilization and radiolabelled DNA hybridization.

2.1.5.1. Southern hybridization.

The DNA to be transferred was developed on an agarose gel as previously described in section 2.1.2.1. The gel was examined under long wave UV-light and trimmed to contain only the required tracks. The gel was then photographed under short wave UV-light. Depurination was

achieved as follows: The gel was gently shaken in 250ml of hydrochloric acid (0.25M) for 15 minutes. The solution was then decanted and the step repeated. Alkaline denaturation was achieved as follows: The gel was gently shaken in 250ml of sodium chloride (1.5) + sodium hydroxide (0.5) for 15 minutes. The solution was then decanted and the step repeated. Neutralization was achieved as follows: The gel was gently shaken in 250ml of sodium chloride (3M) + TrisHCl (1M pH 8.8). The solution was decanted and the step repeated. The gel was transferred to a nitrocellulose filter by capillary transfer according to Southern (1975). DNA was transferred over-night after which the filter was washed in 2x SSC, and baked in a vacuum dessicator, between two pieces of 3MM paper, at 80°C for 2 hours.

Materials:

SSC, (Hydrochloric acid (0.25M), Sodium chloride (1.5) + sodium hydroxide (0.5), Sodium chloride (3M) + TrisHCl (1M pH 8.8))

2.1.5.2. Colony hybridization.

Isolated colonies were picked onto selective agar plates and allowed to grow under the correct culture conditions. A piece of nitrocellulose filter was cut to the correct size and placed on top of the colonies. The plate was then incubated for a further 2 hours, after which the filter was removed and treated as follows: The filter was immersed in 200ml of sodium hydroxide (0.5M) for 2½ minutes and shaken vigorously to remove cell debris. The solution was decanted and the step repeated. The filter was then immersed in 200ml of TrisHCl (0.5M, pH 8.0) for 2½ minutes, with shaking. The solution was decanted and the step repeated. The filter

was then immersed in 200ml of 95% ethanol for 2} minutes, with shaking. The solution was decanted and the step repeated. The filter was then air dried.

Materials:

(Sodium hydroxide (0.5M), TrisHCl (0.5M, pH 8.0), Ethanol ((95%)).

2.1.5.3. Nick translation of DNA to produce a radiolabelled probe.

Radiolabelled DNA was prepared as follows. The constituents of the reaction mix were added in sequence.

vol. (micro-l).	Solution.
X	DNA (approx. 0.5 micro-g).
15.07-X	Sterile distilled water.
0.5	DNaseI (1/40000 dilution of 1mg/ml).
3	dATP, dTTP, dGTP (1.80M).
2.5	10x NT buffer.
1.43	DNA polymerase (3.5 Units/micro-l)
2.5	dCTP ³² (10micro-CI/micro-l).

The reaction mix was incubated at 14°C for 3 hours after which the reaction was stopped by the addition of 2 micro-l of NT stop buffer. Unincorporated DNA was removed by passing the nick translation mix down a de-salting column according to section 2.1.1.4. Before hybridization the probe was denatured to single strands by boiling for 10 minutes, or by incubation with 10 micro-l of sodium hydroxide (5M) for 5 minutes, followed by the addition of 10 micro-l of tris HCl (2M, pH 7.0).

Materials:

NT. stop buffer, (see reaction mix)

2.1.5.4. Hybridisation of M. xanthus DNA to radiolabelled probe.

The nitrocellulose filter prepared according to the methods above was wetted with 8x SSPE. 10ml of a solution containing hybridization solution:formamide (1:1) was added to the filter and allowed to stand for 5 minutes. The radiolabelled probe was then added in a further 10ml of the above solution and incubated with the filter at 42°C O/N. The filter was washed at 50°C in 150ml of 2xSSC + 0.2% SDS. Allowing 3 changes of wash solution over 2 hours. The filter was then air dried and autoradiographed.

Materials:

SSPE., Hybridization solution, (Formamide).

2.1.6. Solutions required for the manipulation of nucleic acids.

Double T:

20g tryptone

5g sodium chloride

Volume to 1 litre with water; +15g for Double T agar.

After sterilized add supplements (Glucose (0.3%), thiamine (0.0005%), magnesium sulphate (1mM), calcium chloride (10mM)).

DCY:

20g caseitone
2g yeast extract
10ml tris HCl (1M, pH 8.0)
8ml magnesium sulphate (1M)

Volume to 1 litre with water

+15g agar for DCY agar; +7.5g agar for DCY top agar.

LB:

10g tryptone
5g yeast extract
5g sodium chloride

Volume to 1 litre with water

+15g agar for LB agar; +7.5g agar for LB top agar.

Antibiotic solutions:

Ampicillin and Kanamycin were dissolved in SDW then filter sterilized using a 0.2 micron (blue-dot) mini-Sart filter disc, and stored at 4°C. Tetracycline was dissolved in 50% ethanol and stored at -20°C. Chloramphenicol was dissolved in 100% ethanol and stored at -20°C.

TE:

10mM tris HCl (pH 8.0)
1mM EDTA

TM:

10mM tris HCl (pH 8.0)
8mM magnesium sulphate

TES:

50mM tris HCl (pH 8.0)
5mM EDTA
50mM sodium chloride

STE:

25% sucrose
50mM tris HCl (pH 8.0)
5mM EDTA

TBE (10x):

108g trizma base
55g boric acid
9.3g EDTA
Volume to 1 litre with water.

SSC (20x):

3M sodium chloride
0.3M sodium citrate
Adjust to pH 7.0 with NaOH

STET:

8% glucose
0.5% triton X-100
50mM EDTA
50mM tris HCl (pH 8.0)

SSPE:

174g sodium chloride
27.6g Na-di-hydrogen phosphate
7.4g EDTA
Adjust pH to 7.4 with NaOH; volume
to 1L with water.

PI buffer:

10ml sodium chloride (1M)
0.1ml calcium chloride (1M)
0.1ml magnesium chloride (1M)
4ml LB

Volume to 100ml with water.

Lysozyme solution:

10mg/ml lysozyme
0.25M tris HCl (pH 8.0)

Triton lysis mix:

0.1% triton X-100
50mM tris HCl (pH 8.0)
50mM EDTA

Salt-saturated isopropanol:

400ml isopropanol
200ml NTE (NaCl (5M); tris HCl (10mM, pH 8.0); EDTA (1mM, pH
8.0))

Darbyshires reagent:

100g phenol crystals
100ml chloroform
4ml iso-amyl alcohol
0.1ml 8-hydroxy quinoline

Shake with 2 changes of 0.2 volume, 1M tris HCl (pH 8.0)

Shake with 2 changes of TE

Store under TE.

Loading buffers:

Agarose gels:

80% glycerol
2% SDS
0.5% bromophenol blue
0.5% xylene cyanol

Acrylamide gels:

80% glycerol
2% SDS
0.5% bromophenol blue

Nick translation (klenow) buffer (10X).

0.05ml tris HCl (2M pH 7.2)
0.02ml magnesium sulphate (1M)
0.002ml dithiothreitol (1M)
0.001ml BSA (10mg/ml)

Volume to 0.2ml with water. Add dNTP's to 5mM and store in 0.001ml aliquots -20°C

TA buffer (10X).

0.185ml tris acetate (2M pH7.9)
0.132ml potassium acetate (5M)
0.1ml magnesium acetate (1M)

Volume to 1ml with water.

Hybridization mix (2X):

7.5ml SSC (20X)
0.3ml tris HCl (2M, pH 7.4)
1.5ml SDS (10%)
0.3ml salmon sperm DNA (10mg/ml)
5.4ml water

Glycogen:

10mg of glycogen was dissolved in 1 ml of water, and phenol extracted with 0.5ml Darbyshires reagent. After spinning in a microfuge for 5 minutes the aqueous layer was removed and the extraction repeated until no precipitate was seen at the interface after spinning. The aqueous layer was placed in a pre-weighed 1.5ml eppendorf tube, and 1ml of

ethanol was added and mixed. The tube was spun in a microfuge for 10 minutes, the supernatant removed, and the pellet of glycogen dried under a vacuum. A second ethanol precipitation was performed and the glycogen obtained as dried pellet. The tube was reweighed and the remaining glycogen resuspended at 10 mg/ml.

2.2. Techniques for the cultivation of microorganisms.

2.2.1. Growth conditions for the cultivation of E. coli.

Unless stated otherwise E. coli was cultivated on LB. agar, or in LB. broth at 37°C. The alternatives to LB. used in this study were Minimal Media, Double T, and DN., the use of which is described in the relevant section. Aeration of liquid culture was provided by a magnetic follower, or by an orbital shaker. Strains were stored on solid media for up to 3 months before re-streaking was required. Long term storage was achieved by mixing 0.9ml of an O/N culture with 0.1ml DMSO and freezing at -80°C.

Light induction was carried out in 250ml flasks containing 50ml of media. Strains were grown under the appropriate selection at 30°C in a constant temperature growth room, with aeration provided by a magnetic follower. Dark conditions were achieved by covering the flask in aluminium foil. Light was provided by two fluorescent strip lights (warm white), the intensity of which was measured from within the flask by a light probe and light meter.

Supplements required: Amino acids were prepared and added according to Davis et al., (1980). Other supplements were prepared as below and included protoporphyrin IX, and delta-aminolevulinic acid. In the

solubilization of protoporphyrin IX two methods were used in this study: Tween80 gave more constant solubilization but due to its heterogeneous nature when precisely controlled nutrient environments were required, acid solubilized protoporphyrin IX was used.

Materials:

LB., DN., Double T, Mineral media, protoporphyrin IX, delta-aminolevulinic acid.

2.2.2. Nitrosoguanidine (NTG) mutagenesis of E. coli.

A 5ml overnight culture of E. coli was diluted 1/50 in fresh media and grown to an OD₆₆₀ of 0.7. The cells were pelleted and washed twice with citrate buffer (0.1 M, pH 5.5). The washed cells were resuspended to an OD₆₆₀ of 0.15 in citrate buffer containing 50 micro-g/ml NTG. Incubation times were varied between 10 and 80 minutes, after which the cells were pelleted and washed twice in phosphate buffer (0.1 M, pH 7.0). The cells were grown in selective media, or in LB. before selection.

Materials:

LB, (Citrate buffer (0.1 M, pH 5.5); phosphate buffer (0.1 M, pH 7.0).

2.2.3. Growth conditions for the cultivation of M. xanthus.

Unless stated otherwise M. xanthus was cultivated on DCY. agar, or in DCY. broth, at 33°C. The alternative to DCY. used in this study was A1, the use of which is described in the relevant section. After colonies had developed on DCY agar the plate was transferred to an 18°C incubator

for 18 days, after which the strain must be re-streaked onto a fresh plate. Long term storage was achieved by mixing 0.9ml of an O/N culture with 0.1ml DMSO and freezing at -80°C . Vigorous aeration was required for growth in liquid culture, and was provided by an orbital shaker, or by a magnetic follower. The culture was insulated from the heating effect of the magnetic stirrer by a layer of polystyrene.

Light induction was carried out in 500ml flasks containing 250ml of media. Strains were grown under the appropriate selection at 30°C in a constant temperature growth room. Light and dark conditions were as for section 2.2.1.

Materials:

DCY., A1.

2.2.4. Materials required for the cultivation of microorganisms.

DN (Defined nutrient):

100ml	A buffer (10X)
1ml	thiamine (0.5%)
1ml	magnesium sulphate (1M)
10ml	glucose (20%)

Sterilize solutions separately and add to 890ml sterile distilled water.

Add solutions after autoclaving 890ml SDW + 15g agar for A1 agar.

Add amino acids for auxotrophy as required.

Add 1ml of vitamin solution, and 10ml trace element solution.

Double T:

20g tryptone
5g sodium chloride

Volume to 1 litre with water; +15g for Double T agar.

After sterilized add supplements (Glucose (0.3%), thiamine (0.0005%),
magnesium sulphate (1mM), calcium chloride (10mM)).

DCY:

20g casitone
2g yeast extract
10ml tris HCl (1M, ph 8.0)
8ml magnesium sulphate (1M)

Volume to 1 litre with water

+15g agar for DCY agar; +7.5g agar for DCY top agar.

LB:

10g tryptone
5g yeast extract
5g sodium chloride

Volume to 1 litre with water

+15g agar for LB agar; +7.5g agar for LB top agar.

Mineral media:

100ml A buffer (10X)
1ml thiamine (0.5%)
1ml magnesium sulphate (1M)
10ml glucose (20%)

Sterilize solutions separately and add to 890ml sterile distilled water.

Add solutions after autoclaving 890ml SDW + 15g agar for A1 agar.

Add amino acids for auxotrophy as required.

A Buffer:

105g di-potassium hydrogen phosphate
45g potassium di-hydrogen orthophosphate
10g ammonium sulphate
5g sodium citrate (di-hydrate)

Volume to 1 litre with water

A1:

5ml tris HCl (2M, pH 7.6)
0.8ml magnesium sulphate (1M)
0.01ml calcium chloride (1M)
0.1ml iron chloride (100mM)
0.5g ammonium sulphate
5g potassium aspartate
0.05g leucine
0.1g isoleucine
0.1g phenylalanine (L-type only)
0.1g valine
0.01g methionine

Volume to 975ml with water, or water agar (1% w/v)

After autoclave add the following components sterilised separately:

1ml vitamin B12 (1 mg/ml)
1ml spermidine HCl (125 mg/ml)
25ml sodium pyruvate (20%)
1ml potassium phosphate (1M, pH7.6)

Trace element solution:

200mg iron chloride (hexa-hydrate)
40mg zinc chloride
10mg copper chloride (di-hydrate)
10mg manganese chloride (quadra-hydrate)
10mg sodium borate (deca-hydrate)
10mg ammonium molybdonate (quadra-hydrate)

Volume to 1 litre with water

Sterilize by filtration.

Vitamin solution:

100mg riboflavine
100mg nicotinamide
10mg p-amino benzoic acid
50mg pyridoxine HCl
50mg thiamine HCl
20mg biotin

Volume to 100ml with water.

Sterilize by filtration, or autoclave at 115°C for 10 minutes, and store in the dark at 4°C.

Antibiotic solutions:

Ampicillin and Kanamycin were dissolved in SDW then filter sterilized using a 0.2 micron (blue-dot) mini-Sart filter disc, and stored at 4°C. Tetracycline was dissolved in 50% ethanol and stored at -20°C. Chloramphenicol was dissolved in 100% ethanol and stored at -20°C.

Protoporphyrin IX (acid solubilized):

Dissolve protoporphyrin IX in 10M HCl. When in solution dilute x2 with sterile water. After addition of required volume to media add an equal volume of 5M potassium hydroxide.

Protoporphyrin IX (Tween 80 solubilized):

Dissolve protoporphyrin IX in 5ml of tween 80. Slowly dilute to 50ml with sterile water. Filter sterilize into 1.5ml aliquots and store at -20°C in the dark.

Delta-aminolevulinic acid:

Dissolve delta-aminolevulinic acid in water to required concentration and filter sterilize. Store at 4°C.

2.3. Techniques for the assay of protein content and enzyme activity.

2.3.1. The assay of beta-galactosidase activity from E. coli.

Beta-galactosidase activity was assayed according to Miller 1972. Samples were withdrawn for assay at regular intervals at which times the OD₆₀₀ of the culture was noted. Four 100 micro-l volumes of culture were made up to 1ml in lacZ buffer and stored in a 1.5ml eppendorf at -20°C

until assayed.

The assay was performed as follows: Each sample was lysed by the addition of two drops of chloroform and one drop of SDS (0.1%) and vortexing the mixture. The tubes were placed at 28°C for 10 minutes, after which 0.2ml ONPG (4mg/ml in lacZ buffer) was added to each tube. After the mixture had developed a pale yellow colour, the reaction was stopped by the addition of sodium carbonate (1M) and the time of incubation with ONPG noted. The OD₄₂₀ and OD₅₅₀ were determined for each reaction versus a blank in which the 0.1ml culture was replaced by 0.1ml LB. The number of units of beta-galactosidase was determined according to the following equation.

$$\frac{1000 \cdot OD_{420} - (1.75 \times OD_{550})}{t \times v \times OD_{600}}$$

Where t is the time of incubation with ONPG in minutes, and v is the volume of culture assayed (0.1ml).

Materials:

lacZ buffer, sodium carbonate (1M), ONPG (10mg/ml in lacZ buffer, SDS (0.1%), chloroform).

2.3.2. The assay of beta-galactosidase activity from M. santhus.

Samples for assay were withdrawn from culture at regular intervals. The volume withdrawn was judged by the optical density of the culture, and ranged from 8ml to 2ml. The withdrawn cells were pelleted and washed twice with 10ml lacZ buffer. A final pellet of cells was

Materials and Methods.

resuspended in 450 micro-l of lacZ buffer and stored at -20°C until assayed. Four determinations of beta-galactosidase activity were performed on each sample. The volume of the sample used depended on the anticipated activity. Either, 2 x 100 micro-l and 2 x 50 micro-l, for samples with low activity; or, 2 x 50 micro-l and 2 x 10 micro-l, for samples with high activity. The following manipulations were carried out at 4°C. The volume of the assays were made up to 100 micro-l with lacZ buffer. To the 100 micro-l cell suspension 50 micro-l of SDS (0.1%) was added and the mixture vortexed. To this 400 micro-l of ONPG (1mg/ml in lacZ buffer) was added and vortexed. For each sample a no-ONPG blank was determined so that the yellow colour of M. xanthus could be subtracted from the yellow product, ONP, released from ONPG by the action of the beta-galactosidase enzyme. The blank contained the largest volume of cells used in the four duplicates, plus 50 micro-l of SDS (0.1%), and 400 micro-l of lacZ buffer. The reactions were incubated at 37°C. After they had developed a pale yellow colour, the reaction was stopped by the addition of sodium carbonate (1M) and the time of incubation with ONPG noted. The blank for each sample was treated in the same fashion. The OD₄₂₀ was determined for each reaction mix and each blank. The spectrophotometer was zeroed using a solution of 0.1ml DCY, plus 0.8ml lacZ buffer, and 0.05ml SDS (0.1%).

The remaining sample was used to determine the protein content in the sample according to section 2.3.3. The activity of beta-galactosidase in each sample was determined using a computer program written by the author, which is listed in Appendix A. This program was based on the following calculation:

$$\frac{OD_{420}(\text{corrected}) \cdot V(p) \cdot 233.33}{\text{micro-g protein} \quad V(z) \quad t}$$

Materials:

lacZ buffer, (sodium carbonate (1M), ONPG (1mg/ml in lacZ buffer, SDS (0.1%))

2.3.3. Assay of protein content (Lowry).

Protein content was assayed according to the method of Lowry et al., (1951). A 5 micro-l and a 10 micro-l volume of cells from the 450 micro-l sample were assayed for protein content. The volume was made up to 150 micro-l with water, to which 150 micro-l of sodium hydroxide (1M) was added. The protein solution was then boiled for 5 minutes and allowed to cool on ice. To the denatured protein solution 750 micro-l of solution C, which should be prepared fresh, was added, mixed thoroughly and allowed to stand at room temperature for 10 minutes. 150 micro-l of Folin-Ciocalteu (50% with water) was added, mixed thoroughly, and allowed to stand at room temperature for 30 minutes before determining the OD₇₅₀. A standard curve of bovine serum albumen (BSA.) (1 mg/ml) diluted to give values between 5 micro-g and 150 micro-g was performed with each series of assays.

Materials:

Solution C, (Folin-Ciocalteu reagent (50% with water), BSA (1 mg/ml))

2.3.4. The use of chromogenic substrates in agar plates.

5 Bromo-4-chloro-3-indolyl-beta D galacto-pyranoside (X-gal) was dissolved in dimethyl formamide to 20mg/ml and stored at -20°C. In top agar supplementation was at 30 micro-l per 3ml. In bottom agar X-gal solution was diluted 1/500.

Isopropyl beta-D thio galactopyranoside (IPTG) was dissolved in water to 24 mg/ml and stored at -20°C. In top agar supplementation was at 20 micro-l per 3ml. In bottom agar IPTG was diluted 1/400.

4 methyl umbelliferyl beta-galactoside (MUG) was dissolved in dimethyl sulphoxide to 2mg/ml and stored at -20°C. The MUG solution was sprayed onto the agar surface containing the colonies of interest and allowed to stand for 5-10 minutes before visualisation under long wave UV.

2.3.5. Materials.

lacZ buffer:

0.06M di-sodium hydrogen phosphate

0.04M sodium di-hydrogen phosphate

0.01M potassium chloride

0.001M magnesium sulphate

0.05M beta-mercaptoethanol

Volume to 1 litre with water.

Adjust pH to 7.0, do not autoclave.

Solution C:

50ml sodium carbonate (5%)

1ml sodium/potassium tetrates (2%)

1ml copper sulphate (1%)

2.4. Techniques for the isolation and analysis of carotenoids.

2.4.1. The extraction of carotenoids from biological materials.

The method of extraction of carotenoids was that of F. Murillo (pers. comm.), modified to incorporate the precautions in handling carotenoids outlined by Britton (1985). A volume of cell culture, routinely 20ml, was pelleted, washed with 10ml phosphate buffer (0.1M, pH 7.0) and pelleted again. The pellet was stored in this form, or lyophilized, at -20°C until assayed. Carotenoid extractions were performed on ice and in the dark where possible. The cell pellet was extracted with either methanol, or acetone:methanol (7:2) for 1 hour. The cell debris was removed by centrifugation and re-extracted until no further pigment was removed. The supernatants were pooled and evaporated under a stream of nitrogen to achieve the required volume; routinely 1-2ml, or to dryness. In some experiments to aid the extraction of carotenoids the cell extractant was briefly sonicated. When carotenoids were contained in methanol alone a partition between methanol and petroleum ether may be performed to facilitate a clearer analysis of the extractant. The colourless intermediate carotenoids are transferred to the petroleum ether fraction, the coloured sugar bound carotenoids remain in the methanol.

Materials:

(phosphate buffer (0.1M, pH 7.0), acetone, methanol, petroleum ether)

2.4.2. Determination of carotenoid content of extractant.

Several methods were used to examine the carotenoids contained in the cell extractant:

2.4.2.1. Spectrographic determination.

Spectra were determined between 300nm and 570nm using a Phillips UV/VIS scanning spectrophotometer PU 8720. Scans were performed in the extractant solutions, methanol, and petroleum ether, or the sample was evaporated to dryness and resuspended in hexane. The latter was used as a standard for comparison with previous tables of carotenoid peaks of absorption (Foppen, 1971).

2.4.2.2. Thin layer chromatographic determination.

The thin layer chromatogram (TLC) plates used were obtained from MERCK, and consisted of aluminum sheets coated with silica gel 60 F₂₅₄, measuring 20x20 cm and 0.2mm thickness. The carotenoid sample was concentrated by evaporation of the solvent(s) under nitrogen, and loaded onto the TLC as a spot of no more than 5mm diameter on a marked baseline. The TLC was developed using acetone:petroleum ether (80:40) in a standard chromatogram chamber. The solvent front was monitored and its position marked when the TLC was removed from the developing solvents. The TLC was air dried for 10 minutes and examined under visible and UV-light.

2.4.2.3. Aluminium oxide column determination.

The column was prepared as follows: The column was made in a glass capillary with dimensions 15cm X 1.5cm diameter. A known weight of aluminium oxide was ground with water (0.8% and 0.3% were used) in a mortar and pestle. The activated aluminium oxide was suspended in

petroleum ether and poured slowly into the column. Several volumes of PE were passed through the column to allow the aluminum oxide to settle evenly. The column was not allowed to dry out at any point and was kept in the dark while developing carotenoids. The carotenoid sample was layered on top of the column, run into the column with a small volume of PE., and developed using a number of different fractions. Each different fraction was collected and stored separately. In sequence these were 1) 5ml PE. 2)10ml PE. 3)10ml PE. 4) 10ml PE. + 10% acetone. The fractions were evaporated to dryness in a rotary evaporator under vacuum at 48°C. The resulting pigment was resuspended in 2ml hexane and determined spectrographically.

Materials:

(Aluminium oxide, petroleum ether (40-80 fraction), acetone, hexane.)

2.4.2.4. High performance liquid chromatographic determination.

The carotenoid samples were developed using isocratic nonaqueous reversed phase high performance liquid chromatography as previously described by Nelis and Leenbeer (1983). The liquid chromatograph used was equipped with a constant flow pump a sample valve with a 20 micro-l loop and a diode array wavelength detector. The column used was a Techsphere ODS 5 micro-m. The mobile phase consisted of acetonitrile: methanol:dichloromethane (40:50:10). Only HPLC-grade solvents were used, these were de-gassed by sonication for 2 hours. The column was operated at ambient temperatures, a flow rate of 1ml/minute, and pressure of 1.45-1.5 kpsi. In repeat experiments the eluent was monitored at visible wavelengths (380, 425, 450, and 470nm), and at UV wavelengths (283, 315,

340, and 380nm). Peak analysis was performed where appropriate to determine peak purity. Columns were eluted for 40 minutes; previously Nells and Leenheer (1983 and 1989) had used 16-30 minute elutions to develop a number of carotenoids of varying chemical composition.

Materials:

(HPLC-grade acetonitrile, HPLC-grade methanol, HPLC-grade dichloromethane)

2.5. Techniques for the isolation and analysis of porphyrins.

2.5.1. The extraction of porphyrins from biological materials.

The method of extraction of protoporphyrin IX from M. xanthus was as described by Burchard and Dworkin (1965) in their initial experiments implicating protoporphyrin IX as the possible photoreceptor/sensitizer. A 200ml culture was grown under appropriate conditions to lag phase. The cells were pelleted and washed once in phosphate buffer (0.1M). Porphyrins were extracted from the cells by glacial acetic acid:concentrated hydrochloric acid (98:2) at 4°C O/N. The cell residue was removed by centrifugation and the pH of the supernatant adjusted to precisely 3.5 with concentrated ammonium hydroxide. To this 1 volume of water was added and the pigment was extracted into ether by shaking with an equal volume of ether for 1 hour at 4°C. Pigments were directly partitioned into hydrochloric acid (1M, or 0.1M), for spectrographic determination; or vacuum evaporated to dryness at 55°C for esterification as described in section 2.5.2.

2.5.2. Methyl esterification of porphyrin pigments.

The extracted pigments were evaporated to dryness as described above (section 2.5.1.). The presence of water will inhibit the esterification process. The method of esterification is that described by Schwartz *et al.*, (1947). To the pigment was added 100ml methanol:sulphuric acid (20:1) which was incubated at 4°C O/N. 300ml of ethyl acetate was added, and the mix was washed with 400ml ice-cold sodium hydroxide (2%), this wash was rapid as the presence of acid or alkali causes saponification of the porphyrin ester. The mixture was washed with 100ml sodium chloride (7%). This step was repeated four times. The mixture was filtered through ethyl acetate moistened filter paper, and vacuum distilled to dryness in a rotary evaporator at 55°C. The residue was resuspended in 1.5ml chloroform, and determined on a TLC. according to section 2.5.3.

2.5.3. Thin layer chromatographic determination of porphyrin methyl esters.

The methyl esterified porphyrins were developed on thin layer chromatograms according to the method of Chu *et al.*, (1951), with the exception that toluene was substituted for the carcinogen, benzene. The thin layer chromatograms used were obtained from MERCK, and consisted of aluminium sheets coated with silica gel 60 F₂₅₄, measuring 20x20 cm and 0.2mm thickness. The porphyrin methyl esters were loaded as a spot of no more than 5mm onto a marked base-line. The porphyrins were developed using a standard chromatogram chamber. The solvent used was benzene(or toluene):ethyl acetate:methanol (83:13.5:1.5) (Doss, 1989). The solvent front was monitored and its position marked when the TLC was

removed from solvent. Pigments required for further identification were scraped from the TLC, and suspended in chloroform in which spectrophotometric analysis was performed.

2.6. List of strains.

2.6.1. List of E. coli strains.

CGSC 4806	<u>hemA41</u> <u>relA1</u> <u>spoT1</u> <u>metB1</u> (Hfr)
C600	F ⁻ <u>thi-1</u> <u>thr-1</u> <u>leuB6</u> <u>lacY</u> <u>tonA21</u> <u>supE44</u>
ED8812	F ⁻ <u>thi-1</u> <u>thr-1</u> <u>leuB6</u> <u>lacY</u> <u>lacZ(M15)</u> <u>tonA21</u> <u>supE44</u>
<u>MC1061</u>	<u>hdsR</u> ⁻ <u>hdsM</u> ⁺ <u>araD139</u> Δ (<u>ara</u> <u>leu</u>)7697 <u>ΔlacX74(lacIPOZY)</u> <u>galU</u> ⁻ <u>galK</u> ⁻ <u>strA</u>
pop2239	F ⁻ <u>thi-1</u> <u>thr-1</u> <u>leuB6</u> <u>lacY</u> <u>tonA21</u> <u>supE44</u> <u>malA510</u>
PR1	<u>hemA41</u> <u>relA1</u> <u>spoT1</u> <u>metB1</u> <u>trpB(tn5(tet^R))</u> (Hfr)
PR2	<u>hdsR</u> ⁻ <u>hdsM</u> ⁺ <u>araD139</u> Δ (<u>ara</u> <u>leu</u>)7697 <u>ΔlacX74(lacIPOZY)</u> <u>galU</u> ⁻ <u>galK</u> ⁻ <u>strA</u> <u>trpB(tn5(tet^R))</u>
PR3	<u>hemA41</u> <u>hdsR</u> ⁻ <u>hdsM</u> ⁺ <u>araD139</u> Δ (<u>ara</u> <u>leu</u>)7697 <u>ΔlacX74(lacIPOZY)</u> <u>galU</u> ⁻ <u>galK</u> ⁻ <u>strA</u>
PR5	PR3 [NTG mutagenised, permeable to protoporphyrin IX]
PR6	<u>hemA41</u> <u>hdsR</u> ⁻ <u>hdsM</u> ⁺ <u>araD139</u> Δ (<u>ara</u> <u>leu</u>)7697 <u>ΔlacX74(lacIPOZY)</u> <u>galU</u> ⁻ <u>galK</u> ⁻ <u>strA</u> <u>malA510</u>

2.6.2. List of M. xanthus strains.

DK101	wild-type
DK1050	wild-type
MR135	<u>carR6 car-12 Δ(carQRS)</u>
MR148	<u>carB1::Tn5-132</u>

2.6.3. Description of plasmids.

Only the features of interest to this study are described.

pDAH217 Contains 5.6 kb Hind III/Bgl II fragment of p^{QRS} region controlling the expression of a promoter-less lacZ gene.

pDAH274 Promoter probe vector containing a promoter-less lacZ gene, and a unique Eco RI site into which foreign promoters may be cloned.

pDAH288 pDAH217 plus the 5.6 kb Stu I fragment containing the carQRS region.

pDAH289 pDAH217 plus the 5.6 kb Stu I fragment containing the carQRS region in the opposite orientation to pDAH288.

pDAH266 Contains 1.2 kb Sma I/Bgl II fragment of p^{QRS} region controlling the expression of a promoter-less lacZ gene.

pDAH266 pDAH266 plus the 5.6 kb Stu I fragment containing the carQRS region.

pDAH287 pDAH266 plus the 5.6 kb Stu I fragment containing the carQRS region in the opposite orientation to pDAH266.

pDAH328 Contains 0.4 kb Sac I/Bgl II fragment of p^{QRS} region controlling

the expression of a promoter-less lacZ gene.

Ref. for all pDAH vectors D. Hodgson pers. comm.

- pMB500 Contains fragment of Mx8 bacteriophage genome including all DNA necessary for integration into the host genome (L. Shimkett pers. comm.).
- pMTL25P Contains large Pst I bound polylinker (Chambers et al., 1988).
- pPL378 Contains carotenoid gene(s) cloned from Erwinia herbicola (Tuveson et al., 1988).
- pHC78 Control plasmid for pPL378, lacks carotenoid genes (Tuveson et al., 1988).
- pIC20R pUC derived plasmid containing large polylinker.
- pOM41 Contains defective promoter region of malPC operon, and unique Eco RI site into which promoters may be cloned (Raibaud et al., 1984)

Plasmids constructed in this study:

- pPR101 pDAH328 plus the 5.6 kb Stu I fragment containing the carQRS region.
- pPR102 pDAH328 plus the 5.6 kb Stu I fragment containing the carQRS region in the opposite orientation to pPR101.
- pPR103 pIC20R plus 1.2 kb Sma I/Bgl II fragment of p^{QRS} region cloned into Hind III (filled)/Bgl II sites.
- pPR104 pOM41 plus Eco RI bound p^{QRS} from pPR103.
- pPR105 pDAH274 plus Eco RI bound p^{QRS} from pPR103.
- pPR108 pOM41 plus Eco RI bound p^{QRS} from pPR103, showing tetracycline resistance.

- pPR107 5.6 kb Xho I Mx8att fragment cloned into the Xho I site of pMTL25P.
- pPR108 5.6 kb Pst I bound Mx8att fragment from pPR107 cloned into the Pst I site in the kanamycin resistance gene of pDAH286.
- pPR110 5.6 kb Pst I bound Mx8att fragment from pPR107 cloned into the Pst I site in the kanamycin resistance gene of pDAH286.
- pPR111 5.6 kb Pst I bound Mx8att fragment from pPR107 cloned into the Pst I site in the kanamycin resistance gene of pDAH287.

CHAPTER 3. A study of the carQRS region from M. xanthus expressed in a heterologous E. coli system.

3.A.1. The use of E. coli as a heterologous host to study biological systems.

The large number of techniques available in the study of E.coli, and the numerous mutants isolated, make it a very attractive heterologous host. E. coli has been used previously to study expression from heterologous DNA, such DNA has been shown to contain sequences which act as promoters in E. coli (eg. Casadaban and Cohen, 1980). E.coli has also been used in the study of a wide range of physiological phenomena, including many of relevance to this study. For example Erwinia caratovorae carotenoid genes have been cloned into E. coli where they have been shown to synthesise carotenoid pigment, which protects the host cell from near-ultraviolet radiation (Lee and Liu, 1991; Perry et al., 1986; Tuveson et al., 1988). E. coli strains containing the hema lesion are defective in the enzyme glutamyl tRNA transferase. hema strains are unable to synthesise porphyrins and ultimately heme and hence cytochromes which are essential for aerobic respiration. These strains when grown in the absence of delta-aminolevulinic acid show increased resistance to near-UV-light (Peak et al., 1987; Tuveson and Sammartano, 1988). This implicates a porphyrin or subsequent product in the sensitizing of cells to near-UV. The ability of E. coli to grow both aerobically and anaerobically has been used to implicate oxygen in the inactivation of cells by UV-light (Peak et al., 1983).

3.A.2. The possible benefits of using a heterologous host.

There are two specific benefits to be gained from the use of E.coli in this study. Firstly, it is hoped to use the p^{QRS} promoter to control genes of industrial importance in other heterologous systems. This could be best done through the development of an easily transferrable DNA "cassette". The conditions required for the induction of this "cassette" having been determined previously in the heterologous host. Secondly, it is proposed that oxygen plays a key role in the inductive process. M. xanthus is not capable of anaerobic growth, however, E.coli is a facultative anaerobe and thus could allow examination of promoter expression in the absence of oxygen.

3.B.1. The use of the lacZ gene as a reporter in biological systems.

Silhavy and Beckwith (1985) have produced an excellent review of the history and uses of the lacZ gene in the study of biological phenomena, of which a summary of the relevant points is presented here.

There are several features of the lacZ gene which have made it so successful in the study of various biological systems. 1) A large portion of the amino terminus can be removed without eliminating potential enzyme activity. 2) There are several indicator media available to identify cells utilizing lactose, this facilitates the identification of clones containing the lacZ gene. 3) The product of the lacZ gene, the enzyme beta-galactosidase is easily assayable and provides a very sensitive means to study gene regulation. 4) The lac operon is one of the most extensively studied genetic systems.

Historically the first lac fusions were created by deletions which fused the promoterless lac operon to the promoters of closely linked genes. To improve the usefulness of the lacZ gene it was fused to phage Mu which provided a means to transpose it at random about the E. coli genome. Subsequent improvements utilized a chimera of phage Mu and phage lambda to allow the easy isolation of target genes into which the lacZ gene had been transposed. Later, plasmid vectors were developed in which restriction sites had been engineered to separate the promoter or promoter and translation initiation signals of the lacZ gene from the coding region allowing precise fusion of the controlling elements of a target gene to the structural region of lacZ.

The lacZ gene fusion has found many uses. It may be used to study the regulation of genes or operons, or to detect genes which are subject to a particular regulatory control. It may be used to localize proteins in the cell and to assay proteins for which no assay exists. Finally it may be used to detect a gene for which a phenotype is known, but for which there is no simple method to identify or clone.

The lacZ gene provides a readily identifiable reporter with which to identify and clone regulatory elements controlling gene expression, and allows identification of mutants which would prove difficult to select by other means. For instance promoter inactivation mutants may be easy to identify but promoter constitutive mutants may give no measurable phenotype unless fused to lacZ. lacZ fusions may be used to monitor mutants in promoter and operator regions, and can be used to distinguish between transcriptional and translational levels of control. Insertion of lacZ into a gene provides a means of studying the regulation of a gene in the absence of its product. This is desirable in some systems for instance

autogenously controlled genes. In some cases a gene may be isolated and sequenced before its physiological role has been fully determined. lacZ fusions to such a gene allow its expression to be studied, and in higher organisms eg. Drosophila information as to a genes tissue specificity and temporal control may be determined. lacZ protein fusions also aid protein analysis facilitating the monitoring of proteins in PAGE gels and by antibodies raised to beta-galactosidase. Gene fusions may be isolated by use of affinity chromatography matrices which recognize and bind the beta-galactosidase component of the fusion. Once obtained, antibodies may be raised against the protein of interest to facilitate the isolation of the native protein. It is the use of lacZ fusions as sensitive and accurate reporters of gene expression that is of central importance to the present study. M. xanthus does not contain a native beta-galactosidase activity and there are a wide range of mutants of E. coli available which are deleted for the lacZ gene. Strain MC1081 which is deleted for the entire lac region was used in this study.

3.B.2. The use of single copy vectors in the study of regulation in biological systems.

The initial use of multicopy plasmid vectors may be justified in their ease of use and manipulation in establishing the regions of DNA warranting further investigation. However, multicopy plasmids present a number of problems in the study of regulatory mechanisms. Overproduction of certain proteins may be toxic to the cell and cause compensatory mutations in the DNA of interest. Quantification of gene expression is difficult due to possible variation in copy number as determined by a number of factors,

eg. growth phase and media composition (Raibaud et al., 1984 and therein). The presence of numerous copies of the same DNA sequence may titrate limiting factors required for the correct regulation of this sequence. The latter is exemplified in the studies of Sadler et al., 1977, in which it was shown that the presence on plasmids of an increasing number of chemically synthesised lac operators increased the deregulation of the genomic lac operon.

To overcome these problems, which may or may not be relevant to this study, the DNA of interest was fused to the malPQ operon. The product of this operon amylomaltase shows a number of advantages described above for beta-galactosidase, including ease of assay, which is of primary importance. Similarly it has been shown that it is possible to manipulate the DNA of the promoter controlling the malPQ operon whilst maintaining expression (Debarbouille and Raibaud, 1983). This allows the insertion of convenient cloning sites into the promoter region allowing foreign DNA to be cloned with ease in place of the malPQ promoter. Using the technique described by Raibaud et al., 1984; and Vidal-Ingigliardi and Raibaud, 1985, the target DNA may be integrated into the host chromosome in single copy. The technique allows the DNA fragment of interest to be inserted into an engineered Eco RI fragment in the malP promoter, which is contained on a plasmid. The plasmid contains regions of homology with the E. coli chromosome and strains may be selected in which the plasmid has recombined with the genome placing the DNA of interest in single copy into the host genome. The DNA is thus placed upstream of the malPQ operon so that if it contains a promoter this promoter now drives through the malPQ operon.

3.C.2. Plasmids used in this study.

Three different restriction fragments, centred on the region identified as the p^{QRS} promoter (Figure 9), were used in promoter probe vectors. These fragments were cloned into a pACYC derived plasmid such that the p^{QRS} promoter controlled the expression of a promoter-less lacZ gene (D.A.Hodgson pers. comm.).

All regulatory elements identified so far in the carQRS region may be excised on a 5.8 kb Stu I fragment. Derivatives of the promoter probe plasmids previously described were constructed containing this Stu I fragment in either one orientation, or the other. As such these plasmids contain the p^{QRS} promoter controlling the lacZ gene, and also the carQRS region controlled by a second p^{QRS} promoter. The set of plasmids was completed in this study by cloning the 5.8 kb, fragment containing the carQRS region into a plasmid containing the Bgl II/Sac I promoter region (Figure 10).

3.1. The expression of p^{QRS} promoter probe plasmids in E. coli(MC1081).

3.1.1. The cloning of high copy number p^{QRS} promoter probe plasmids.

The cloning scheme for the insertion of the 5.8 kb Stu I bound carQRS region fragment is shown in Figure 10. A 2.8 kb Cla I(Bac I) fragment containing the Bgl II/Sac I promoter fragment was isolated from pDAH328 and ligated to a 15.7 kb Cla I(Bac I) fragment from both pDAH288 and pDAH289; these contain the carQRS region in alternative orientations. Ligation of vector and insert regenerates a functional kanamycin resistance gene, and provides a positive selection for chimeric

Figure 8. The regions of the p^{QRS} promoter from which the three fragments cloned into the promoter vectors were derived. , \blacktriangle indicates the transposon insertion site in omega-DK1910, and omega-DK1911. Sm) Sma I, S) Sac I, B) Bgl II, H) Hind III. From Hodgson (1987).

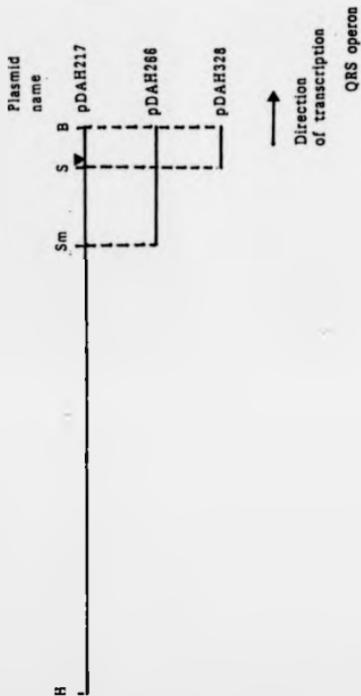
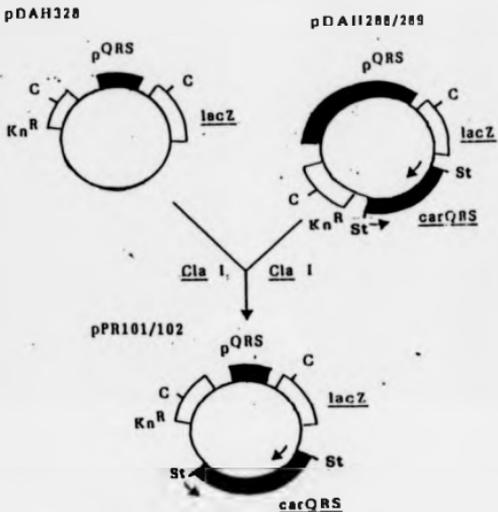


Figure 10. Cloning scheme for the insertion of the 5.8 kb Stu I fragment of the carQRS region into pDAH328 in both orientations. Plasmids pDAH288/289 differ in the orientation of the fragment containing the carQRS region, designated by opposing arrows. C) Cla I. St) Stu I



DNA. The presence of insert and its orientation were confirmed by a Cla I and a Pst I digest respectively. The orientation of the 5.6 kb fragment was checked by an Sph I digestion. In pPR101 the p^{QRS} promoter in the 5.6 kb carQRS region reads away from the Kanamycin resistance gene, in pPR102 it is reversed.

3.1.2. p^{QRS} promoter expression from high copy number plasmids in E.coli.

The strain of E. coli used in this study was MC1081 which carries a deletion of the lac region. Two different p^{QRS} expression profiles were seen in this strain:- Firstly plasmids containing the Hind III/Bgl II (pDAH217, 286, and 287) promoter fragment and Sma I/Bgl II (pDAH286, 288, and 289) promoter fragment will be considered (Figure 11). Secondly plasmids containing the Sac I/Bgl II (pDAH328, pPR101, and 102) fragment (Figure 12).

3.1.2.1. Expression of 5.8 kb (Hind III/Bgl II) and 1.2 kb (Sma I/Bgl II) p^{QRS} fragments in E.coli.

Typical examples of promoter expression profiles seen in MC1081 from the two larger fragments (pDAH217, 286, 288, 287, 288, and 289) are shown in Figure 11. Insertion of the promoter containing fragment is seen to result in a 4 fold increase in lacZ activity, compared to plasmids which lack the promoter insertion. The introduction of the entire carQRS region on a 5.6 kb fragment resulted in a 2 fold decrease in promoter activity. This decrease is seen to be independent of the direction in which the carQRS fragment was inserted. However, when the carQRS region is

Figure 11. Promoter activity from the 1.2 kb fragment of p^{ORS} in E. coli. ▼) 1.2 kb fragment, ◆) 1.2 kb fragment in the presence of the carQRS region. ○) no 1.2 kb fragment, or carQRS region.

Figure 12. Promoter activity from the 0.4 kb fragment in E. coli. ◆) 0.4 kb fragment, ○) 0.4 kb fragment in the presence of the carQRS region oriented such that the p^{ORS} promoter drives in opposition to the 0.4 kb fragment, △) 0.4 kb fragment in the presence of the carQRS region oriented in the opposite direction to above.

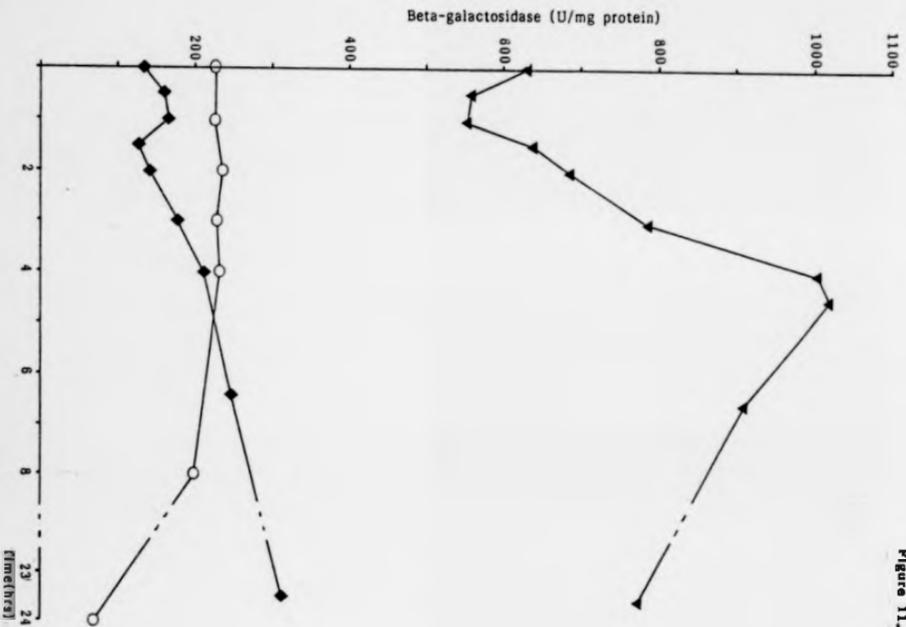


Figure 11.

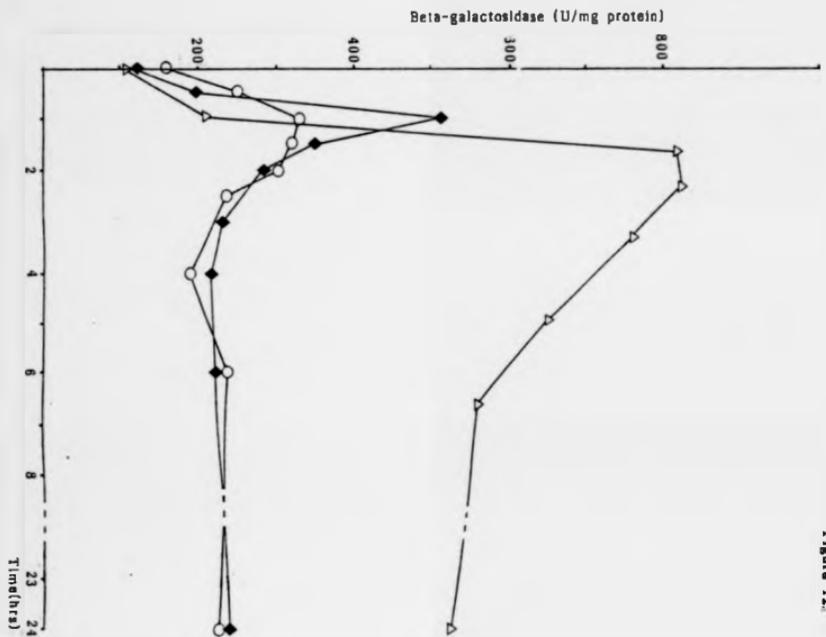


Figure 12.

orientated in the same direction as the promoter fragment driving lacZ the promoter activity measured is consistently approximately 100 Units less than when it is in the opposite orientation.

3.1.2.2. Expression of the 0.4 kb (Sac I/Bgl II) p^{QRS} fragment in E.coli.

Plasmids containing the Sac I/Bgl II promoter fragment showed decreased levels of promoter activity in E. coli (MC1081) (Figure 12), at around 250 Units, as compared to approximately 800 Units seen with the larger promoter fragments. Expression from this smaller fragment showed an orientation specific activation in the presence of the entire carQRS region. pPR102 in which the carQRS region is reading towards the kanamycin gene, shows increased promoter activity. It should be noted that the profiles of all expression curves of the 0.4kb promoter fragment show a similar shape, peaking after 1-2 hours followed by a decrease in activity. Expression curves of the two larger promoter fragments show a bell shape, which is flattened in the presence of the carQRS region.

To test if the reduced activity seen with the 0.4 kb promoter fragment was due to a promoter mutation, plasmid pDAH328 was transduced into M. xanthus using a high titre, phage P1 transduction (see Methods). Low phage titres, which are routinely used to transduce M. xanthus did not transduce at a high enough frequency to allow recombinational insertion of the plasmid via the small region of homology between the plasmid and the chromosome. The resulting strain (DK101[pDAH328]) has two copies of the carQRS promoter (see Figure 12), the genomic copy drives the lacZ gene on the plasmid, the plasmid derived copy drives the carQRS region and consequently controls the production of

carotenoids. Plasmid integration was confirmed by Southern Blot analysis. Wild type, light inducible carotenoid synthesis was seen, and lacZ activity was similar to DK101[pDAH217], (results not shown).

3.1.3. The effect of light on p^{QRS} expression from multicopy plasmids in E.coli(MC1081).

The initial results obtained with the large fragments from the p^{QRS} promoter region were encouraging. It appeared that the expression in E. coli was of a similar type to that seen in M. xanthus; namely that the promoter is expressed to a high level, which is repressed in the presence of the entire carQRS region. In M. xanthus the p^{QRS} promoter may be induced by exposure to light, the effect of which was studied in E. coli. All promoter probe plasmids expressed in strain MC1081 were tested under light (80 micro E m⁻² s⁻¹) and dark conditions. The light conditions used in this experiment were tested using M. xanthus (DK101[pDAH217]) which showed a 25 fold increase in promoter expression as measured by lacZ activity, compared to a dark grown culture. There was no detectable difference in promoter activity from any promoter probe plasmid, when comparing expression under light and dark growth conditions.

3.1.4. The effect of plasmid size on promoter expression.

Initial results were promising in that the large promoter fragments gave what appeared to be a similar activity to that seen in M. xanthus. Promoter activity in the presence of the entire carQRS region could not be induced by a light treatment. Whilst this was disappointing, it suggested

that E. coli may have great potential in determining the requirements for p^{QRS} induction in that reconstitution experiments could now be performed. However, the true nature of the repression needs to be determined as the possibility arises that one of a number of mechanisms may be causing the repression seen. Possible mechanisms which do not involve repression of promoter activity by a product of the carQRS region, include a decrease in plasmid stability caused by the increase in size. It may be envisaged that the doubling in size caused by the introduction of the carQRS region may place a significant burden on the cell selecting for either down regulated mutants or maintenance of the plasmid at a lower copy number. The possibility that it is the effect of increased plasmid size that is causing the decrease in promoter activity seen may be examined by introducing a 5.8kb piece of DNA into the promoter probe vectors in place of the carQRS region.

A control plasmid (pPR108) was constructed (described in Chap.4). This plasmid contained the 1.2 kb promoter fragment driving the lacZ gene. In addition it contained a 5.8 kb fragment from the mycobacterial bacteriophage, Mx8, cloned into the ampicillin resistance gene in a similar position to the 5.8 kb carQRS region, described above. Promoter activity from this plasmid (pPR108) in E. coli was identical to that from plasmid pDAH266 (also containing the 1.2 kb promoter fragment), and 2 fold higher than plasmid pDAH286 (containing the entire carQRS region (results not shown)).

3.1.5. The production of a mal⁻ E.coli strain using plasmid pOM41.

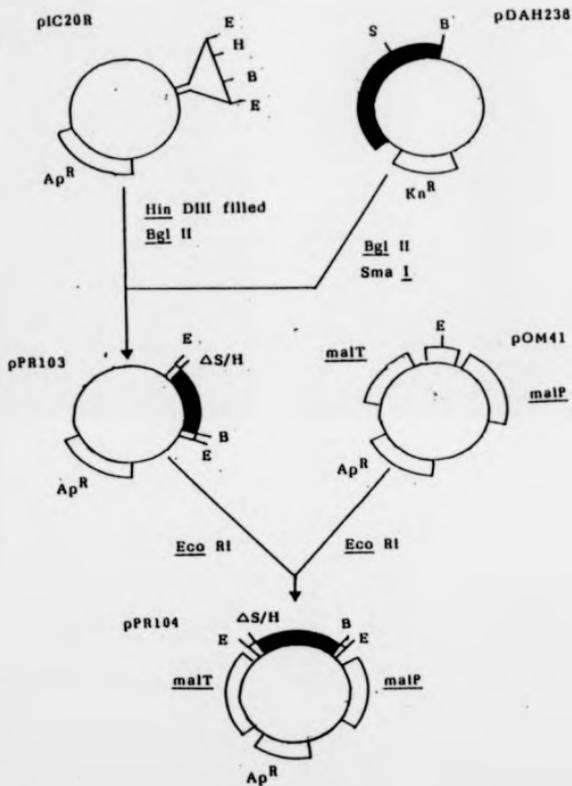
The method of Raibaud et al. (1984) to insert promoter probe plasmids into the E. coli host genome was used in this study. The principle of this method is discussed in Chapter 3.B.2. To test the ease with which interchange is achieved a defective malP promoter was transferred into E. coli strain PR3. PR3 was constructed to examine the effect of increased photosensitizer on promoter expression, (see Section 3.2.). A mal⁻ version of PR3 was produced in order that single copy plasmids could be used in the photosensitizer study. This strain was produced according to previously described protocols (Raibaud et al., 1984) and was named PR8.

3.1.8. The cloning of single copy number promoter probe plasmids into E. coli.

The limitations of multicopy plasmids have been discussed previously (3.B.2.). In order to limit such possible errors the p^{QRS} promoter region was cloned into the pOM41 vector which would allow its expression to be monitored in single copy. pOM41 contains a defective malP promoter; thus after homologous recombination transferring the plasmid bound promoter region into the chromosomal malPQ operon, it is the p^{QRS} promoter that will control expression from the operon.

The cloning strategy used to insert the p^{QRS} promoter into plasmid pOM41 is illustrated in Figure 13. The pOM41 plasmid contains a single Eco RI site into which target DNA may be cloned. To facilitate this the p^{QRS} promoter was cloned into an Eco RI bound polylinker. A 1.2 kb Sma I/Bgl II fragment, from pDAH238, containing the carB promoter was

Figure 13. Cloning scheme for the insertion of ρ^{QRS} into pOM41. B) Bgl II; H) Hin DIII R) Eco RI; S) Sma I.



cloned into pIC20R digested with Hind III, and filled using the "klenow" fragment (Methods 2.1.1.6.), then digested with Bgl II. The klenow filled end was thus compatible with the blunt end generated by Sma I.

The ligation mix was transformed into E.coli (ED8812) and chimeric DNA identified via alpha-fragment complementation using X-gal. Insertion of DNA into the polylinker destroys the plasmids ability to complement the host lesion, therefore the colonies remain white, on media containing containing X-gal and IPTG.

Insert size and orientation were determined by Pvu II and by Sac I digests respectively. A plasmid of the correct structure was named pPR103.

3.1.7. The cloning of the polylinker bound p^{ORS} promoter into plasmid pOM41.

The 1.2kb Eco RI bound promoter fragment was isolated from pPR103 and ligated into similarly cut pOM41. Transformants of E. coli strains C800 and pop2239 were selected on ampicillin. Insert orientation was confirmed by both Bam HI and Pst I digestion. The Pst I digestion required the mapping of a previously unmapped Pst I site in the mal region at 9 kb on the plasmid. A plasmid of the correct structure was identified and named pPR104.

Insertion of a promoter in the correct orientation into the Eco RI site in plasmid pOM41, places the promoter-less tet^R gene, downstream of this site, under the control of the incoming fragment. Tetracycline resistance could not be obtained from any plasmid irrespective of promoter orientation. Plasmid pPR104 was used to generate Mal⁺ cells in pop2239. pop2239 is a Mal⁻ strain, containing a defective p^{malPQ}, homologous

recombination between the plasmid and the chromosomal p_{malPQ} regions should introduce the cloned promoter into the chromosome. If this promoter is active a Mal^+ phenotype is seen. Mal^+ colonies were obtained at the same frequency as an untransformed strain, indicating them to be natural revertants.

It is possible that cloning the promoter fragment upstream to the tetracycline resistance gene is a lethal event, and thus selection for loss of the plasmid, or down regulation of the promoter is being selected for. Over-expression of the tetracycline gene has previously been shown to result in loss of membrane potential and cell death (Eckert and Beck, 1989). For this reason polylinker bound promoter activity was tested under non-lethal conditions in a lacZ promoter probe plasmid (pDAH274), transformed into MC1081. This places the promoter upstream of a promoter-less lacZ gene, allowing its activity to be assayed without the requirement for the promoter to be active for the survival of the cells. The orientation of the insert with respect to the lacZ gene was by a Bam HI digest. A plasmid of the correct orientation was termed pPR105. Solid media tests of beta-galactosidase activity using X-gal and MUG were inconclusive, suggesting little or no beta-galactosidase activity was being expressed by the plasmid. Quantitative assays were performed which revealed that promoter insertion in either the correct or incorrect orientation yielded beta-galactosidase activity two-fold lower than vector plasmid alone. Expression from this vector plasmid is in turn two-fold lower than that of pDA11208 (containing the 1.2 kb promoter fragment).

All cloning procedures described in sections 3.1.6. and 3.1.7. were repeated. This yielded identical results in all cases except one tetracycline

resistant colony was isolated in strain C800, this plasmid was termed pPR106.

3.1.8. Examination of the tetracycline resistant phenotype seen in plasmid pPR106.

The expression of tetracycline resistance by a polylinker-bound promoter fragment was the desired phenotype, showing an active promoter had been placed and maintained in front of the tetracycline resistance gene. As such the promoter fragment giving this phenotype was investigated further.

The tetracycline resistance character was shown to be stably maintained when selecting for ampicillin only. The insert could be isolated on an Eco RI fragment of the correct size, and the promoter was shown to be in the correct orientation within the polylinker through a Mlu I/Hind III digestion. An attempt was made to transfer the resistance phenotype to another E. coli strain, and to transfer the promoter to the pDAH274 promoter probe plasmid. A large-scale preparation of plasmid DNA was performed selecting for tetracycline resistance. The 1.2 kb promoter fragment could not be isolated from this preparation and a Pst I digest revealed a deletion of approximately 1.3 kb in the region of promoter insertion.

Mini-preparations were performed on the original clone and it was shown that the 1.2 kb fragment could only be isolated from plasmid in cells grown under ampicillin selection. Thus it appears that the strain contains two different forms of the plasmid. One in which the insert has been deleted placing the tetracycline gene under the control of some other

promoter, which allows expression of the tetracycline resistance phenotype. The second in which the insert is intact. The latter form is lost under tetracycline selection. Thus, this plasmid is of no further use in this study, however, it does indicate that selection for deletants in the promoter fragment cloned in front of the tetracycline gene is occurring, and that tetracycline selection is promoting the loss of insert.

3.2. The effect of increased levels of photosensitiser on p^{ORS} expression in E.coli.

3.2.A. The production of an E. coli strain capable of protoporphyrin IX uptake.

The heme synthesis pathway has been elucidated by the isolation of a number of mutants in the various structural genes (Cox and Charles, 1973; Sasarman et al., 1975; McConville and Charles, 1979(a); Sasarman et al., 1979). One of these mutants, deficient in the hemA gene product, was used in this study. This mutation renders the cell unable to synthesise delta-aminolevulinic acid (d-ALA). The auxotrophy may be satisfied by the addition of d-ALA. The hemA lesion was originally thought to be in the enzyme d-ALA synthase. However it has been subsequently shown that E. coli synthesise d-ALA exclusively via the glutamate pathway rather than glycine. Subsequently the hemA lesion was identified as being a deficiency in glutamyl-tRNA-transferase (Avissar and Beale, 1989).

A hemA mutant is unable to form functional cytochromes (Haddock and Schairer, 1973), and consequently cannot perform aerobic (oxidative) respiration (Ingledeu and Poole, 1984). The loss of cytochromes and consequently the ability to respire oxidatively may be shown by the

addition of glycerol which may be respired oxidatively but is too reduced to be respired fermentatively, and so cannot be utilized by a hemA strain. The presence of this lesion was used as the basis for the selection of a protoporphyrin IX uptake mutant. Protoporphyrin IX biosynthesis occurs after the hemA lesion in the heme-synthesis pathway. However, E. coli is impermeable to protoporphyrin IX. Consequently, growth on media containing glycerol as the sole carbon source and supplemented with protoporphyrin IX may be due to one of two events. 1) Reversion of the hemA lesion, revertants can be identified by growth on media lacking d-ALA, growth of hemA strains being prevented on this media; 2) The uptake and incorporation of protoporphyrin IX into the heme synthesis pathway. Similar uptake mutants have been isolated previously which were selected by permeability to haemin (McConville and Charles, 1979(b)), however these mutants have subsequently been lost from culture collections.

3.2.1. Bacteriophage P1 transduction to produce progenitor strain.

Having established a high level of promoter activity in E. coli, and its repression in the presence of the carQRS region, a concurrent experiment was initiated to determine the effect of increased levels of the proposed photosensitizer, protoporphyrin IX, on promoter activity in E. coli. To do this, a strain capable of the uptake of protoporphyrin IX from exogenous media was constructed.

The characters of importance in the E. coli strains used in this construction are as follows: Strain CGSC4808 carries a lesion in the hemA gene; strain NK7408 carries a Tn10 insertion in the trpB gene. The

trpB::Tn10 gene is closely linked to the hemA gene and thus the Tn10 insertion provides a linked selectable marker for the hemA gene. Strain MC1081 carries a deletion of the lac operon. All strains are described fully in Methods 2.8.1..

Strain CGSC4808 was found to grow on minimal media supplemented with delta-aminolevulinic acid in the presence of glucose (fermentative) and glycerol (non-fermentative) as sole carbon source. In the absence of delta-aminolevulinic acid, glycerol cannot be utilized and only poor growth is achieved on glucose, due to the relative inefficiency of fermentation, compared with aerobic respiration.

Two different routes were employed to transfer this lesion in the hemA gene into MC1081.

In the first construction the hemA lesion was tagged with tet^R. The tet^R gene in strain NK7408 was transduced into strain CGSC4808, using virulent form bacteriophage P1. Tetracycline resistant transductants were screened for a requirement for tryptophan and delta-aminolevulinic acid in minimal media; and for phage sensitivity. A transductant showing the correct phenotype was named PR1.

The tet^R marker was in turn transduced from PR1 into MC1081. Despite repeated attempts, transductants were not observed. It was noted that citrate at 10mM, used in selection plates, inhibited cell growth, therefore, this concentration was reduced to 1.7mM. NK7408 did not show this inhibition due to citrate, suggesting the hemA lesion is contributory to this effect.

Increased expression time did not aid the selection of transductants; this was not unexpected since the tet^R gene is substrate inducible. Phage titre was increased from 1×10^7 used previously, to 3×10^{10} . Using this high

titre phage, 18 positive transductants were obtained, 4 of which showed a requirement for delta-aminolevulinic acid and tryptophan in minimal media. One example was named PR4. An alternative bacteriophage, T4, was also employed. Using this method 98 tet^R colonies were isolated, all of which showed a requirement for delta-aminolevulinic acid and tryptophan.

Had the second approach not yielded the required strain, a fusidic acid resistant strain of PR4 would have been selected. In such a strain the transposon will have been lost, making the strain more stable by removing the danger of spurious transposition.

The alternative construction involved the production of an intermediate strain PR2. The transposon in NK7408, was P1 transduced into MC1081 selecting for tetracycline resistance. An example of a phage sensitive, tet^R, trp::Tn10, lac⁻, transductant was called PR2. The trp gene from CGSC4808, was P1 transduced into PR2, selecting for trp⁺ colonies on minimal media. The resulting 8 transductants were screened for the requirement for delta-aminolevulinic acid. A transductant showing the following phenotype: Hema⁻, Lac⁻, Tet^S, Trp⁺, was termed PR3.

Fortuitously a second transduction appears to have occurred in the construction of PR3. This strain is now lou⁺, ara⁺.

3.2.2. NTG. mutagenesis of the progenitor strain (PR3) and the screening for a protoporphyrin IX uptake phenotype.

Preliminary experiments were performed to determine if a protoporphyrin IX uptake mutant could be isolated via non-mutagenic selection. A large inoculum of the progenitor strain PR3 was used to seed a minimal media liquid culture containing protoporphyrin IX. In addition a

large number of exponentially growing PR3 cells were washed and streaked onto minimal plates solidified with agarose and containing protoporphyrin IX. These methods did not yield any colonies that could be used in further studies.

A kill curve was produced according to Methods 2.2.2. which showed that exposure of the progenitor strain PR3 to NTG at 50 micro-g/ml for 35 minutes resulted in 50% cell death, which is the recommended value for this mutagen (Adelberg *et al.*, 1965). Occasionally to enhance a "leaky" phenotype a second round of mutagenesis must be performed.

The progenitor strain was mutagenised to 50% cell death. The resulting mutagenised stock was grown in liquid minimal media containing protoporphyrin IX with glycerol as the only carbon source. Aliquots were withdrawn at 24, 48, and 72 hours post-mutagenesis. This was in order to examine the cultured cells before possible revertants swamped any uptake mutants whilst still allowing the maximum time to express any possible genetic lesions. The withdrawn aliquots were spread plated onto agarose solidified minimal media containing protoporphyrin IX. Two types of colonies were seen: large colonies which were either buff, or pale brown in colour; and small dark brown colonies which were considerably fewer in number. A representative selection was picked and screened for the retention of the hamA lesion and the ability to take up protoporphyrin IX. Eight colonies were selected which fulfilled these criteria, these eight which included examples of both colony types were tested for growth in liquid media.

Growth in liquid minimal media containing protoporphyrin IX and with glycerol as the only carbon-source resulted in very poor growth in the first 50 hours, followed by an increase in growth rate (results not shown). This

letter increase was accompanied by the accumulation of hemA revertants.

The proposed uptake mutants were grown in LB media supplemented with protoporphyrin IX. The mutants were able to grow to an OD₆₈₀ of 1.0 in around 12 hours. The progenitor strain, PR3, showed an increase of 0.35 over the same time period, and both strains showed some growth in unsupplemented LB.

The mutants were grown in minimal media containing casamino acids (0.2%) and a vitamin solution (0.1%) (Methods 2.2.4.). This was termed Defined Nutrient (DN.) media. In solid form this gave an all-or-nothing differentiation between protoporphyrin IX supplemented and unsupplemented media when tested with the uptake mutants. The growth curve of mutant 4A is shown in Figure 14. This shows an increase in OD₆₈₀ in protoporphyrin IX supplemented media of around 1.4 OD units; whereas in unsupplemented media no growth was seen. Figure 15 shows the growth of PR3 in identical media. The progenitor strain is able to grow to a small extent in porphyrin supplemented media. This growth was dependent on the porphyrin and was not caused by the Tween 80 which was used to solubilize it (results not shown). The mutant 4A was termed PR5 and was used in all subsequent studies.

The uptake mutant PR5 exhibited an unusual phenotype. When transferred from protoporphyrin IX supplemented media to delta-amino-levulinic acid supplemented solidified media the resulting colonies where of a marked red colouration.

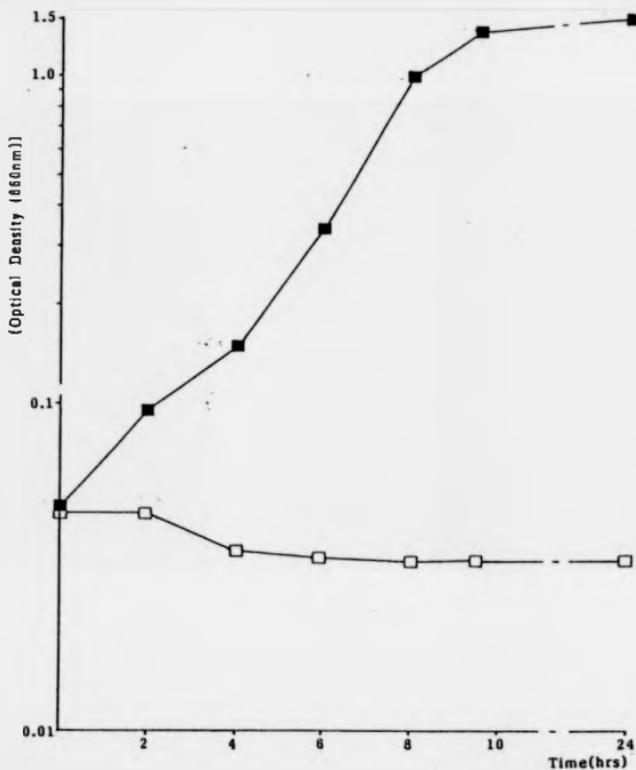


Figure 14. The growth of PR5, the protoporphyrin IX uptake mutant in DN media both supplemented with protoporphyrin IX ■, and unsupplemented □.

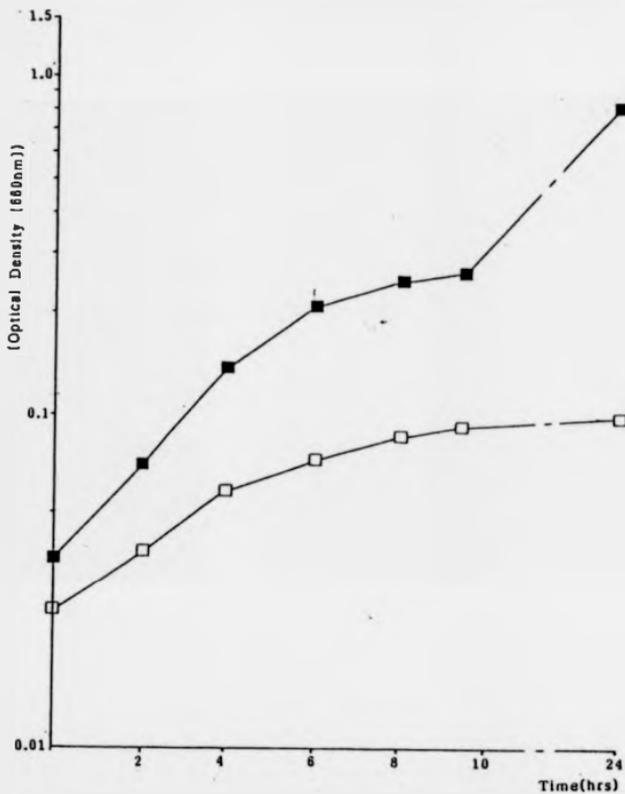


Figure 15. The growth of PR3, the progenitor strain to PR5, in DN media both supplemented with protoporphyrin IX ■, and unsupplemented □.

3.2.3. p^{QRS} promoter expression in E. coli strain PR5 in the presence of protoporphyrin IX.

Having established a strain capable of the uptake of protoporphyrin IX, promoter activity was determined in this strain in the presence of protoporphyrin IX at a concentration in media of 50 micro-g/ml. A quantitative determination of the extent to which intracellular protoporphyrin IX has increased was not possible since protoporphyrin IX adheres strongly to cell membranes, thus making the distinction between intra- and extracellular protoporphyrin IX difficult.

PR5 was made competent by calcium chloride treatment (Methods 2.1.3.1.) and transformed with plasmids pDAH286, pDAH286, and pDAH287. Transformation frequency was greatly reduced in this strain. Plasmids were reisolated from positive transformants and structures confirmed by restriction digest. p^{QRS} activity was examined in DN. liquid cultures containing protoporphyrin IX. On illumination p^{QRS} activity was seen to decrease (Figure 18). This was also seen in identical cultures in which protoporphyrin IX was replaced by delta-aminolevulinic acid. The decrease seen could not be distinguished from the variation in promoter activity seen throughout the growth cycle of the culture. In many cases where a drop in activity was seen it was accompanied by a decrease in optical density, however, this may have been in part caused by bleaching of protoporphyrin IX. After a 24 hour exposure to light the cultures of PR5 in the presence of protoporphyrin IX did not yield any colonies forming units at any dilution as compared with the dark grown control which contained active bacteria. The effect of light was confirmed by examining p^{QRS} activity from patches of PR5 containing promoter probe plasmids grown on

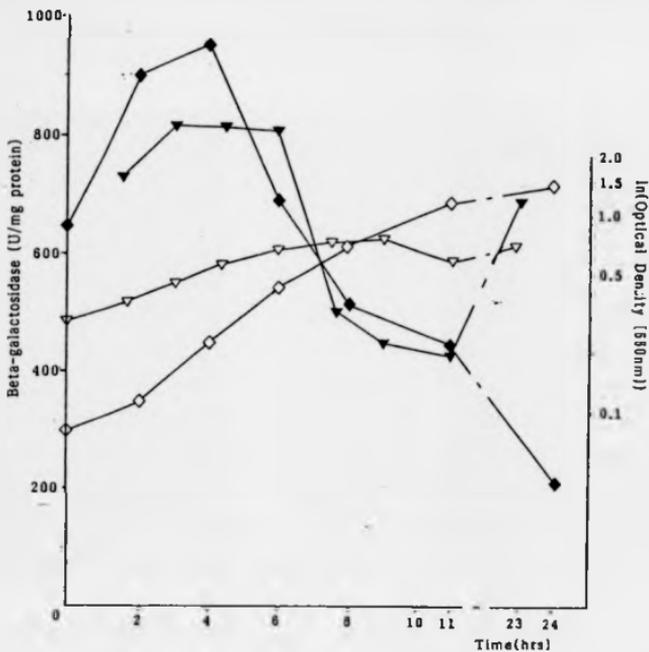


Figure 16. The effect of light and protoporphyrin IX, on the growth of PR5 and the expression of the 1.2 kb promoter fragment. (▽) in A680 PR5 [pDAH268] in media supplemented with protoporphyrin IX, (○) in A680 PR5 [pDAH268] in media supplemented with delta-aminolevulinic acid, (▼) promoter activity from PR5 [pDAH268] in media supplemented with protoporphyrin IX, (◆) promoter activity from PR5 [pDAH268] in media supplemented with delta-aminolevulinic acid.

solid DN. Transfer of plates from dark to light was seen to decrease promoter activity, and growth, as measured by protein assay. Plates transferred from the light to the dark showed some recovery of growth, and an increase in promoter activity (Figure 17).

3.2.4. The effect of carotenoid pigments on the photolysis of the protoporphyrin IX uptake mutant.

Previous results had shown that the PR5 uptake mutant appeared to be photosensitized by protoporphyrin IX in the presence of light. such photosensitivity is seen in M. xanthus, this suggested that the uptake mutant may be used as a model system to study the interaction of photosensitizer and carotenoid. The carotenoid genes of Erwinia herbicola had previously been cloned and shown to protect E. coli from the harmful effects of near-UV (Tuveson et al., 1988). The effect of these genes on the photolysis of PR5 in the presence of protoporphyrin IX was examined. The level of photolysis was determined from a serial dilution determining the number of colony forming units remaining in the culture. PR5 was transformed with pPL378 which contains the carotenoid genes, and with pHC78 which is the plasmid into which the genes were cloned. Positive transformants with pPL378 were seen to be yellow illustrating the synthesis of carotenoids was taking place. Figure 18 shows the effects of light and dark on both strains under identical conditions. In the absence of carotenoid there is a lag of 3 hours before a rapid fall in CFU's is seen. In the presence of carotenoids the number of CFU's begins to fall after 1 hour however the decrease is less rapid. In the absence of carotenoids the decrease in CFU's from 10000 to 100, and from 1000 to 10 takes 2 hours

Figure 17. The effect of light and dark incubations on growth and promoter activity in cells from solid DN. media containing protoporphyrin IX. Promoter activity expressed from plasmids pDAH286, and pDAH286 is shown as measured in cells grown on solid media transferred from light (L) to dark (D), and visa versa. The amount of protein present in the cell sample is shown as a measure of the degree of inhibition of growth by light and protoporphyrin IX.

Figure 18. The effect of carotenoid genes from Erwinia herbicola on the level of photolysis of PR5 in the presence of protoporphyrin IX.

◇) In CFU PR5 [pHC79], no carotenoid, in culture exposed to light;
◆) In CFU PR5 [pHC79], no carotenoid, in culture maintained in the dark; △) In CFU PR5 [pPL378], containing carotenoid gene(s) cloned from Erwinia herbicola, in culture exposed to light; ▲) In CFU PR5 [pPL378], containing carotenoid gene(s) cloned from Erwinia herbicola, in culture maintained in the dark (Tuveson et al., 1978).

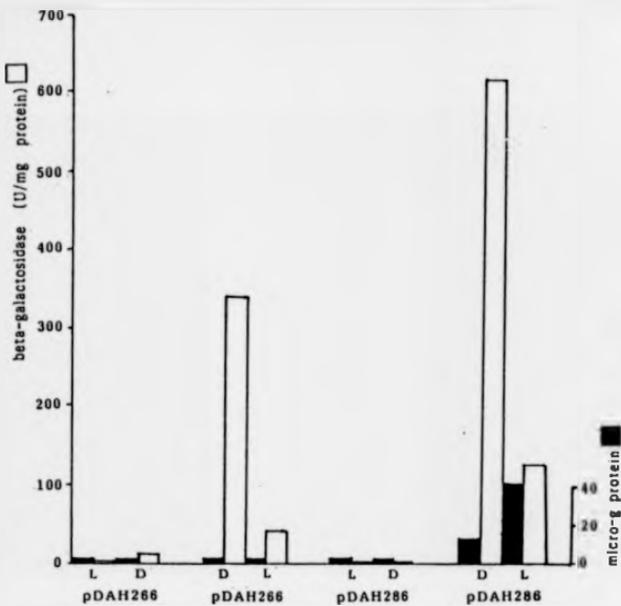


Figure 17.

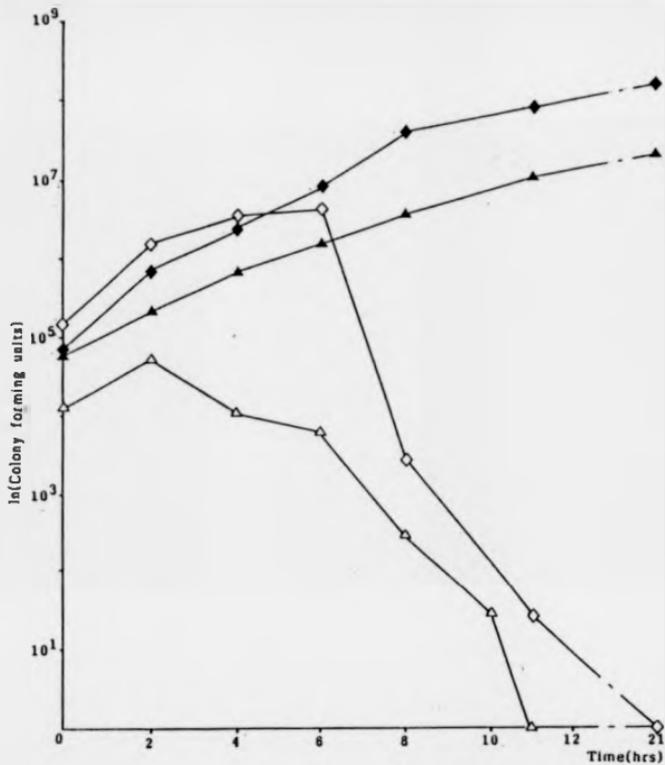


Figure 18.

whereas in the presence of carotenoids the time taken is 31. The significance of this result could not be pursued due to lack of time and awaits further investigation.

3.3. Discussion of results.

3.3.1. Expression of p^{QRS} in E. coli.

Initial results appeared promising, the 5.8 and 1.2kb fragments of the p^{QRS} region appeared to contain a promoter(s) that was expressed at high levels in E. coli. This expression was reduced by the introduction of a 5.8kb fragment containing the structural genes of the carQRS region. The insertion of 5.8kb of unrelated DNA could not reproduce the apparent repression seen with the carQRS region.

The 0.4kb p^{QRS} fragment exhibits much less promoter activity in E. coli than do the 5.8kb, and 1.2 kb fragments. Initially it was assumed that the minority result was the rogue. That the 0.4kb promoter fragment was defective in some way. The increased activity seen from pPR102, which contains the 0.4kb fragment and the carQRS region oriented such that it is driving in the same direction as the reporter gene could be explained as being due to read through from the carQRS region driving through the stem loop terminators present upstream of the 0.4 kb promoter fragment. Such terminators are known to be less than 100% efficient. If this were so it would be expected that read through would also occur in plasmids containing the larger promoter fragments. However, it could be argued that the presence of the repressor binding to an operator present in the larger promoter fragments would serve to terminate any such read through.

However, further analysis showed that the 0.4 kb fragment was fully

functional in M. xanthus. Subsequent analysis has shown that the p^{QRS} promoter lies within the 0.4 kb region (D. Hodgson and S. McGowan pers. comm.) thus it must be assumed that the p^{QRS} expression profile in E. coli is that seen from the 0.4 kb fragment only. The expression seen from the larger fragments is probably due to the presence of a promoter like element, termed (p^X), upstream of the Sac I site at which the E. coli RNA polymerase is binding. However it must be stressed that while the site of transcriptional initiation has been shown (McGowan 1989), the extent of the region of DNA required for full promoter expression in M. xanthus is still being determined (S. McGowan pers. comm.).

Expression from plasmids containing the two larger promoter fragments was repressed by the carQRS region. Sequence analysis of this region has shown that the repressor coded for by the carR gene is most likely to be membrane bound, and does not contain any of the known DNA binding motifs. This raises the question of how such a protein when expressed in E. coli could repress promoter expression. One possible explanation could be that the additional copy of the p^X sequence present on the promoter probe plasmids containing the carQRS region may sequester a limiting factor required for the initiation of expression from p^X. Thus causing an overall decrease in the expression from the copy of p^X linked to the reporter gene. This would thus be seen as a repression. The observation that repression occurs independently to the orientation of the 5.8kb insert makes it unlikely that drive through from the carQRS region into the reporter gene is resulting in the repression seen (for discussion of the action of terminators see Gents et al., 1981).

The ability to express p^{QRS} in single copy may have helped to resolve the problems seen with high copy number vectors. The method of

Raubaud et al., (1984) for inserting DNA in single copy into the E. coli genome was used to transfer a defective mal gene into PR3. This created the required progenitor which after mutagenesis and selection for protoporphyrin IX uptake, would have been used to insert the p^{QRS} promoter in single copy into the genome. However we were unable to clone a functional promoter into the required vectors. The promoter region being used, now termed p^X, is highly expressed in E. coli which may be deleterious to the cell when this promoter is controlling the expression of the tetracycline resistance gene. The product of the resistance gene inserts into the cell membrane (Chopra and Howe, 1978), a large number of insertions may lead to an impairment of membrane function and cell death (Eckert and Beck, 1989), thus selecting for down regulation, and deletion mutants in the p^{QRS} promoter. Such a deletant was isolated in this study (Section 3.1.9).

3.3.2. The effect of light and protoporphyrin IX on E. coli.

The presence of light alone was found to have no effect on the expression of either p^{QRS}, or p^X in E. coli strain MC1061. The activity of the p^X promoter was examined in the presence of light and protoporphyrin IX. Expression was found to decrease reflecting the photolytic nature of protoporphyrin IX in the presence of light. This effect was also seen on solid media, and was due to the presence of protoporphyrin IX, as when the uptake mutant was illuminated in the presence of delta-aminolevulinic acid no such photolysis occurred. An attempt was made to examine p^X expression at sub-lethal light intensities, maintaining protoporphyrin IX as constant (results not shown). Light intensity of 55 microE m⁻² s⁻¹ or less

are required to allow normal growth at which point p^X activity is similar to a dark grown control. This experiment employed differences of 20 $\text{microE m}^{-2} \text{s}^{-1}$ between points which may have missed the crucial crossover point between lethality and biological response.

The nature of the mutation(s) which allow the uptake of protoporphyrin IX into the cell remains to be determined. The nature of the phenotype, and the reduced efficiency of transformation suggest the lesion may have effected the structure of the membrane. Additional evidence to support this comes from the fact that the mechanism of transformation is thought to involve the cell membrane primarily, and it is observed that transformation of PR5 with plasmids is reduced in efficiency. Plasmids that are selected for by ampicillin show further reduction in transformation efficiency. Ampicillin acts at the cell membrane and may exacerbate the effect of the lesion in PR5, whereas kanamycin acts on cytoplasmic constituents and would not have this effect.

PR5 was used as a model system to illustrate the effect of carotenoid genes, cloned from Erwinia herbicola, on photo-sensitization by protoporphyrin IX. It would be expected that the presence of carotenoids should impart upon the cell some degree of photoprotection. The level of photoprotection will vary depending upon the area of the cell in which the photosensitizer is active. The number of CFU's in the culture containing cells expressing carotenoid genes is seen to begin to decrease earlier post light shock, than in the control culture in which the cells do not express carotenoid genes. Possible explanations for this unexpected result include: 1) the carotenoids are acting as photoreceptors and contributing to the photolytic effect; 2) the number of CFU's in the carotenoid containing culture was approximately 2 logs below the culture which lacks carotenoid

at the time when the cells were exposed to light, as such it may be the degree of shading within the culture that is causing the difference seen. If a comparison is made of the time taken for the number of CFU's to fall between set values, at which points shading can be assumed to be the same, it is seen that the presence of carotenoids does render a degree of photoprotection upon the culture.

It is hoped that when the genetic elements involved in the induction of p^{QRS} have been further elucidated using M. xanthus, this work will be pursued to its conclusion. The tools for the study of the effect of protoporphyrin IX on p^{QRS} expression have been established. This combined with the aerobic/anaerobic growth of this organism render it a potentially invaluable means by which to categorically establish the link between light, oxygen, and protoporphyrin IX, in the induction of the p^{QRS} promoter.

The PR5 strain may prove useful to study the effect of chemicals on cell physiology which are normally impermeable to the membrane. Prior to such an investigation the cause of the uptake phenotype may need to be determined.

CHAPTER 4. Light induction of p^{QRS} in M. xanthus, and the genetic analysis of the carQRS region using the Mx8att site as an alternative integration site.

4.1. Light induction of M. xanthus (DK101[pDAH217]).

4.1.A.1. The use of batch culture in the study of carotenogenesis.

Myxobacteria may be cultured in liquid media using artificial media as previously described (Introduction 1.A.1. and Methods 2.2.3.). Well established techniques were used to culture the bacteria in batch. Unless otherwise stated batch cultures were of 250ml of media contained in a 500ml flat bottomed flask with rapid agitation being provided by a magnetic flea. Cultures were grown in a constant temperature growth room at 30°C.

The limitations of batch culture are particularly relevant to the study of physiology. Microorganisms are capable of adapting rapidly to changes in their environment and as such a batch culture represents a constantly varying population. As the cells grow in culture they perturb their environment, if in high enough density this perturbation can result in adaptation of the cells to this new environmental factor (Tempest (1970)). This causes a continuous variation in the physiology of the individual cell in a growing population with time. If these limitations are borne in mind there can be no doubt as to the usefulness of the batch culture in the study of microorganisms.

4.1.1. The isolation of protoporphyrin IX from cells of Mycrococcus xanthus strain DK101.

Protoporphyrin IX has been proposed as the photoreceptor for carotenogenesis in M. xanthus. The M. xanthus strain, DK101 used in this study is a variant of strain FB. The latter strain was used in the studies of Burchard and Dworkin (1988) in which Protoporphyrin IX was implicated as the photoreceptor/sensitizer in carotenogenesis/photolysis. Strain DK101 was derived by multiple passage through liquid culture whilst periodically demonstrating that the strain was still capable of fruiting. This selected for a mutant that clumped less in liquid culture but was still able to fruit (D.A.Hodgson, pers. comm.). This potential difference, and a report that protoporphyrin IX could not be isolated from M. fulvus (H.Reichenbach, pers. comm.) prompted an attempt to confirm the presence of protoporphyrin IX in the DK101 strain.

Protoporphyrin IX was extracted according to the protocol in Methods 2.5.1. The extract was split into two halves, to one of which was added 25 micro-g/ml of a sample of protoporphyrin IX obtained from Sigma. This was to act as a positive control for future manipulations. Both samples were methylated according to Methods 2.5.2., and developed on a thin layer chromatogram using benzene:ethyl acetate:methanol (83:13.5:1.5). A brown spot was seen in the unsupplemented sample at an equivalent Rf to that of the Sigma sample of protoporphyrin IX; both spots were scraped into chloroform, and scanned between wavelengths of 350-700nm. The spectrum of the supplemented extraction showed a large peak at 409nm, and submaxima at 507, 542, 578, and 830. The unsupplemented sample showed a smaller Soret peak, at around 1/10 that seen in the supplemented

sample, and submaxima at 508, 542, 577, and 830. The identity of the compound producing this spectrum was further investigated using characteristic properties outlined by Schwartz et al., (1981). The pigment spot was scraped from the TLC and eluted into di-ethyl ether. Using the large Soret peak as a measure of concentration it was determined that shaking the extract with an equal volume of 5% HCl extracted around 2/3 of the porphyrin. This result is consistent with the sample being Protoporphyrin IX, and distinguishes it from meso-, deuto-, copro-, uro-, and hematoporphyrin.

4.1.2. The accumulation of carotenoids seen after white light induction of p^{QRS} in M. xanthus (DK101(pDAH217)).

Previous experiments had shown that p^{QRS} activity, as measured by the lacZ reporter gene, is increased after exposure to light. This activity peaks then decreases to a constant induced level. Carotenoids may be seen in the induced culture around 2-3 hours after illumination (Hodgson 1987). The point at which the amount of methanol soluble carotenoids increases, and a crude quantitative measure of the increase in carotenoid concentration was determined.

M. xanthus was grown in a 250ml batch culture, in the dark, over night. At an OD₆₈₀ of around 0.15 the culture was exposed to light. p^{QRS} activity as measured by beta-galactosidase activity was assayed according to Methods 2.3.2.. Carotenoid content was assessed in cells pelleted from 20ml of culture and washed twice with 0.1M phosphate buffer (pH 7.0). The pellet was extracted with methanol for 30 minutes at 4°C, cellular debris was removed by centrifugation and the supernatant washed with an

equal volume of petroleum ether (40-60 fraction) for 30 minutes. The absorption spectrum of the methanol layer was determined between 300nm-500nm. The amount of carotenoid was approximated as the area under the curve and expressed per mg of protein in the 20 ml cell pellet. Results are shown in Figure 18. A detectable rise in expression from p^{QRS} is seen 2 hours after exposure of the culture to light. Carotenoid content in a dark grown control culture did not rise above a basal level. The accumulation of carotenoids in cells of the light grown culture is detected around 3 1/2 hours after exposure to light. p^{QRS} activity peaks 8 hours post light exposure, after which it decreases to a constant level. The amount of carotenoid increases almost in parallel with p^{QRS} activity, upto the point where promoter activity decreases. At this point carotenoid content continues to increase. Analysis of carotenoid content in the latter stages of the cultures growth cycle was not performed.

4.1.3. The dose response of p^{QRS} to differing light intensities.

Unless otherwise stated the light intensity used to induce the p^{QRS} promoter in M. xanthus was 120 microE $m^{-2} s^{-1}$. The possibility exists that more than one photoreceptor is involved in the inductive process. This may be detected by studying the induction of the p^{QRS} promoter at different light intensities. The participation of two, or more, photoreceptors is usually, although not always, seen as a bi-phasic (or multi-phasic) dose response curve. The dose response of the p^{QRS} promoter between intensities of 35 microE $m^{-2} s^{-1}$, and 550 microE $m^{-2} s^{-1}$.

M. xanthus was grown in a 250ml batch culture in the dark over night. At an OD_{880} of between 0.1 and 0.15 the culture was exposed to

Figure 18. Light induction of p^{QRS} in M. xanthus DK101[pDAH217] and the subsequent increase in carotenoid content of the induced cells: Δ) p^{QRS} activity from cells in the culture exposed to light, \triangle) p^{QRS} activity from cells in the culture maintained in the dark, \blacklozenge) methanol soluble carotenoids per mg cells in the culture exposed to light, \circ) methanol soluble carotenoids per mg cells in the culture maintained in the dark.

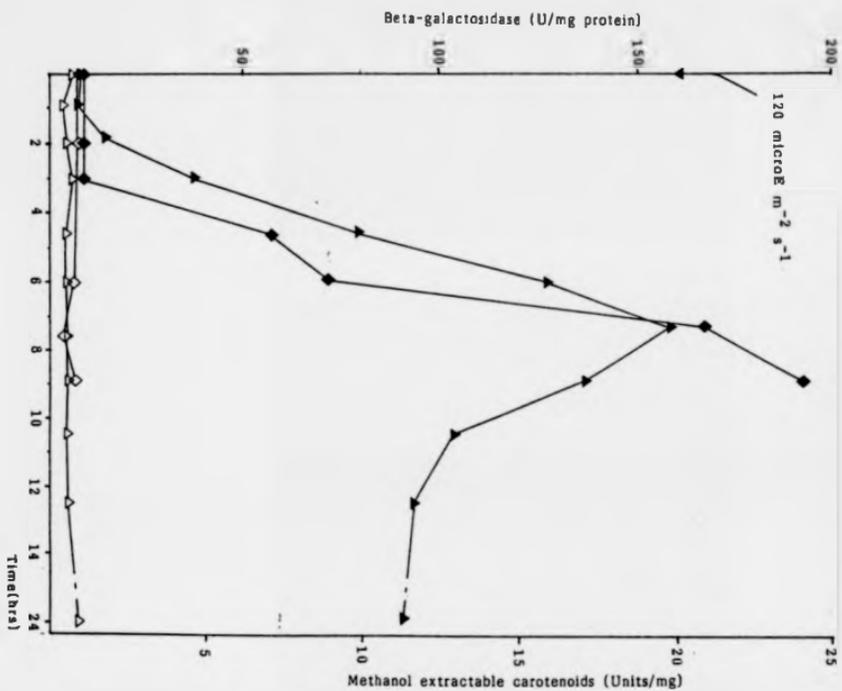


Figure 19.

light. Light intensity was varied by altering the distance and the number of layers of muslin between the light source and the culture. Light intensity was measured from within the culture flask once the experiment was completed. p^{QRS} activity was measured at time 0hr in the dark and every hour upto and including hour 8 post-light induction. Beta-galactosidase was determined according to Methods 2.3.2. and plotted graphically. The areas were plotted graphically and are shown on Figure 20. It can be seen that the response increases linearly between $35 \text{ microE m}^{-2} \text{ s}^{-1}$ to $550 \text{ microE m}^{-2} \text{ s}^{-1}$.

4.1.4. The effect of growth phase on p^{QRS} expression.

The metabolic state of individual cells change throughout the growth cycle of a culture. It would be expected that the growth phase of the culture would exert some effect on p^{QRS} expression measured in cells therein. The extent to which growth phase affects the level of p^{QRS} activity was determined.

M. xanthus was grown as a 750ml batch culture in the dark. At several optical densities: OD_{600} of 0.1, 0.3, 0.5, 1.0, 100ml aliquots were removed, cultured according to methods, and exposed to light at $120 \text{ microE m}^{-2} \text{ s}^{-1}$. p^{QRS} activity was monitored over a six hour period and compared to the original flask which was kept in the dark. Figure 21 shows the initial levels of promoter activity of the cells exposed to light. Expression from the p^{QRS} promoter induced by light at $120 \text{ microE m}^{-2} \text{ s}^{-1}$ decreased 5-fold as the culture aged.

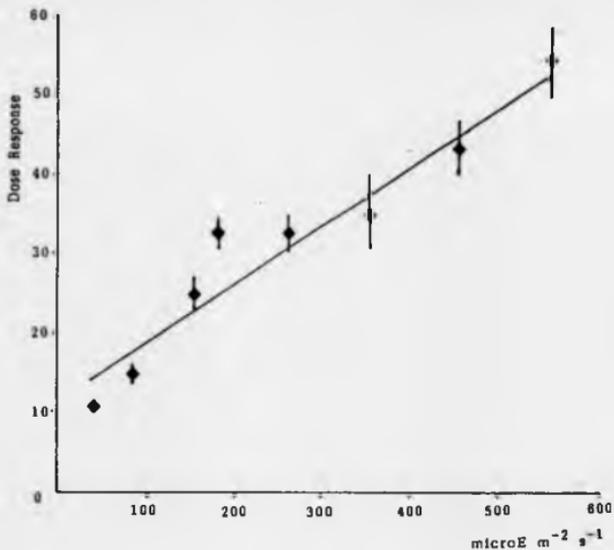


Figure 20. The variation in p^{QRS} activity induced by differing doses of illumination: \blacklozenge) Dose response, calculated from the area under the curve of p^{QRS} induction profiles at different light intensities.

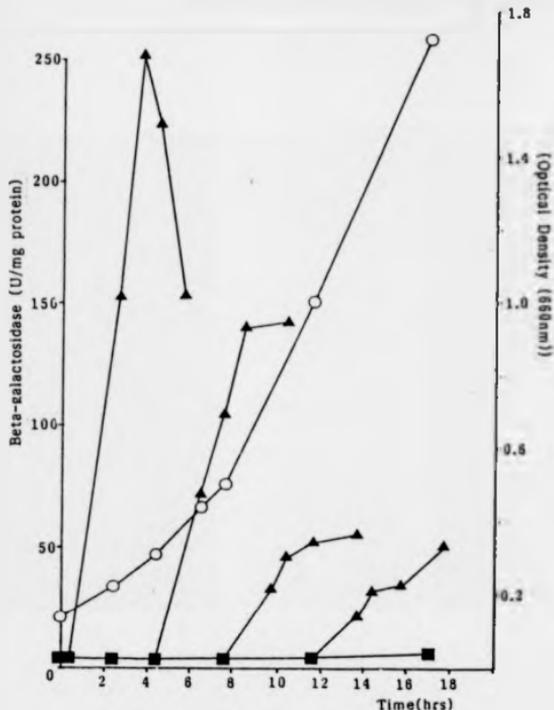


Figure 21. The effect of growth phase on the initial induction of p^{QRS} activity: (○) A_{880} of the culture maintained under dark conditions, (■) p^{QRS} activity in cells from the culture maintained under dark conditions, (▲) p^{QRS} activity from cells removed from the dark grown culture and exposed to identical light treatments. The point at which p^{QRS} activity from the light induced cells bisects the activity of the culture maintained in the dark, indicates the time at which the cells were removed from the dark grown culture and exposed to light.

4.1.5. Defined wavelength light induction of p^{QRS} in M. xanthus.

The work of Burchard and Dworkin (1966) and, Burchard and Hendricks (1969) produced an action spectrum for the accumulation of carotenoid pigment. The accumulation of carotenoids occurs as the final step in a long chain of reactions, any of which may be susceptible to modifications by metabolic or physical parameters which may result in a distortion of the action spectrum. To determine if this were so an attempt was made to produce the action spectrum for the initial event in the accumulation of carotenoids, namely the activation of the p^{QRS} promoter.

4.1.5.1. Using refracted light.

High intensity light ($10000 \text{ microE m}^{-2} \text{ s}^{-1}$) was passed through a monochromator which refracts the light allowing only a small wavelength range to pass in to the illuminated culture. Stray light was removed by light proof cloth and silver foil. The light emerging was at $1 \text{ microE m}^{-2} \text{ s}^{-1}$. In repeated experiments the best differentiation in p^{QRS} activity between dark and defined light at 410nm showed a 4 unit increase in activity in the light (results not shown).

4.1.5.2. Using filtered light.

One possibility for the failure to induce p^{QRS} activity with defined wavelength light may be that the light is of insufficient intensity to produce a detectable change. To overcome this filters which transmit a greater intensity of light of the incident irradiation were used.

High intensity light ($10000 \text{ microE m}^{-2} \text{ s}^{-1}$) was filtered to allow only defined wavelengths to illuminate cultured M. xanthus. This allowed a greater intensity of light to be employed. Distance from the source of light was adjusted so that light of $22 \text{ microEm}^{-2} \text{ s}^{-1}$ emerged from the filters, this was the highest value achievable with all filters. Wavelengths of 408, 439, 483, 547, were used in case the reported maxima of 410nm did not apply to this system. In all cases promoter activity did not vary from that seen in the dark grown control.

4.1.5.3. Using laser light.

To determine whether the lack of induction was due to experimental design, or due to the inability of light of 410nm to induce p^{QRS} activity, very high intensity laser light was used.

To examine the response of the p^{QRS} promoter to defined wavelengths of light at high light intensity a visit was made to the Rutherford Appleton laboratory at Didcot from the 7-11/8/89. A Lumonics pulsed dye laser was used which produced light of precisely 410nm. 250ml batch cultures of M. xanthus were grown in a water bath at 30°C. When at an OD_{680} of between 0.1 and 0.2 cultures were exposed to defined wavelength laser light. Results are shown in Figure 22. Laser light of $40 \text{ microE m}^{-2} \text{ s}^{-1}$ resulted in an increase in p^{QRS} activity from 3-5 units to 10 units over 7 hours. This culture was transferred to the dark overnight and subsequently exposed to laser light at $240 \text{ microE m}^{-2} \text{ s}^{-1}$ which resulted in an increase of 30 Units in p^{QRS} activity over the next 8 hours. At this point the culture had achieved an OD_{680} of 1.75 and was diluted into fresh media. The diluted culture was exposed to laser light of 2400

Figure 22. Induction of p^{QRS} in M. lantus by defined wavelength laser light at 410nm: ▲) p^{QRS} activity; A) culture exposed to 40 microE m⁻² s⁻¹ of defined wavelength light of 410 nm; B) culture returned to dark; C) culture exposed to 240 microE m⁻² s⁻¹ of defined wavelength light of 410 nm; D) culture exposed to 2400 microE m⁻² s⁻¹ of defined wavelength light of 410 nm.

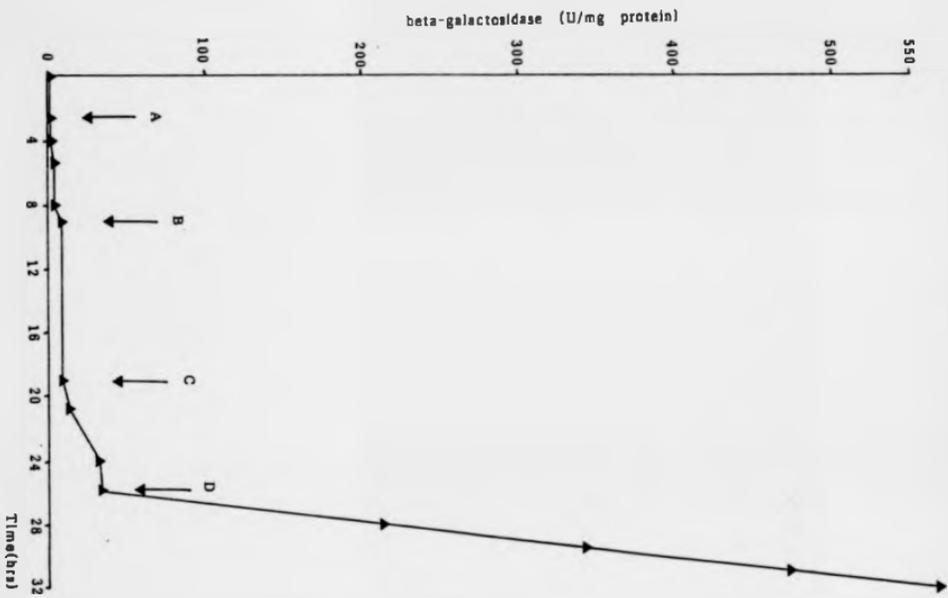


Figure 22.

microE m⁻² s⁻¹ which caused an increase in p^{QRS} activity from 40 units to 585 units over 8 hours at which point the experiment had to be terminated.

4.1.6. The effect of carotenoid on white light induction of p^{QRS} in M. xanthus

Previous results have shown that the decrease seen after the peak in light induced promoter activity is concurrent with the accumulation of methanol extractable carotenoids (4.1.2.). This suggests the possibility that it is the carotenoid molecule that is resulting in the quenching of the light signal resulting in a decrease in p^{QRS} expression. The effect of carotenoid on p^{QRS} activity was determined.

4.1.6.1. White light induction of p^{QRS} in M. xanthus in the presence of exogenous carotenoid.

None of the carotenoids present in M. xanthus are available commercially. As such the most widely available carotenoid, beta-carotene, was used to attempt to quench the light signal by the addition of exogenous pigment. The carotenoid proved difficult to solubilise in aqueous media and formed clumps in the culture with no visible colouration of the media. Addition of beta-carotene had no effect on the induction of p^{QRS} by white light at 120microEm⁻²s⁻¹ (results not shown).

4.1.6.2. White light induction of p^{QRS} in M. xanthus in the absence of endogenous carotenoids.

The M. xanthus strain designated MR148, isolated by Martinez-Laborde (1988), has a Car^- phenotype due to the insertional inactivation of the carB gene. This is currently the only strain available which is deficient in carotenoid synthesis, has a wild type carQRS region, and does not contain a lacZ gene insertion. The carB gene is thought to code for a protein involved in the metabolic pathway before phytoene (Martinez-Laborde et al., 1990). As such it allows the study of p^{QRS} induction in the absence of phytoene and all subsequent carotenoids derived from phytoene. The p^{QRS} promoter probe plasmid, (pDAH217), was introduced into this strain according to Methods 2.1.3.6. A transductant carrying the plasmid was cultured over night to an OD_{860} of 0.18, at which point it was exposed to light of $120 \text{ microE m}^{-2} \text{ s}^{-1}$. MR148 was originally derived from DK1050, therefore expression in the strain MR148[pDAH217] was compared with that in DK1050[pDAH217]. Figure 23. shows the expression of both strains in the light and the dark. There is no difference in the initial induction of p^{QRS} expression after exposure to light. In the presence of the wild-type complement of carotenoids, p^{QRS} activity is seen to peak 7 hours after exposure to light, thereafter promoter activity decreases to a constant reduced level. In the absence of the wild type complement of carotenoids such a peak of expression is not seen, and activity continues to increase, although at a slower rate than was seen in the initial stages of induction.

Figure 23. White light induction of p^{QRS} in the absence of endogenous carotenoids: \blacklozenge) p^{QRS} expression from DK1050[pDAH217], which synthesises a full complement of carotenoids; \diamond) p^{QRS} expression from MR148[pDAH217], which contains a mutation in the carB gene and so cannot synthesise any carotenoids (Martinez-Laborda (1980).

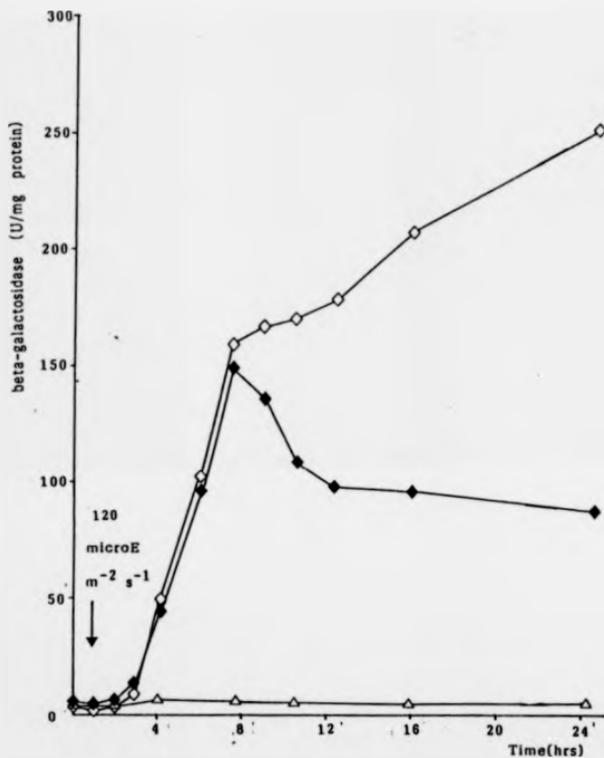


Figure 23.

4.2. Genetic analysis of the carQRS region using the Mx8att site as an as an alternative integration site.

4.2.A.1. The use of an alternative integration site for p^{QRS} promoter probe plasmids.

The plasmids used in this study contain various amounts of the carQRS region. To examine if these regions contain sufficient DNA to allow normal p^{QRS} activity their expression was examined in strain MR135 which contains a 12.98 kb deletion spanning the entire carQRS region. The deleted strain no longer contains DNA with homology to the plasmid so homologous recombination cannot occur. In order to facilitate integration of the plasmid into the host genome the integration system of the Mx8 bacteriophage was employed. The bacteriophage Mx8 is a generalised transducing phage, whose chromosome consists of double stranded DNA (Martin et al., 1978), which at present has been partially characterised (Stellweg et al., 1985). The integrative system has been isolated on a 12.6kb Eco RI fragment of the phage genome. However, the presence of this DNA in M. xanthus may lead to pseudo lytic infection. To avoid this the 5.6kb XhoI fragment containing the integrase enzyme and the attP site and may be substituted for the larger region.

4.2.B.1 Plasmids used in this study.

Plasmid pMB500 is derived from pBR328 and contains the 12.6kb Eco RI region of the Mx8 genome cloned into the Eco RI site (L.Shimkets pers. comm.). Plasmid pMTL25P is pUC derived and contains a large polylinker with a number of useful sites and the partition region to aid

maintenance of the plasmid in culture (Chambers *et al.*, 1988). The p^{QRS} promoter probe plasmids have been described earlier (see Chapter 3.B.2.)

4.2.1. Cloning of the 5.8kb Mx8att fragment into the pMTL25P polylinker.

Initial attempts to clone the 5.8kb Mx8att fragment into the Pat I site within the ampicillin resistance gene of the p^{QRS} promoter probe plasmids by blunting all overhanging ends resulted in large numbers of deletions. This approach was abandoned. The alternative cloning strategy in which the fragment is cloned via the pMTL25(P) polylinker is outlined in Figure 24. The 5.8kb Mx8att fragment was ligated into the Xho I site of pMTL25(P). A plasmid of the correct structure was named pPR107

4.2.2. Cloning of polylinker bound Mx8att fragment into p^{QRS} promoter probe plasmids.

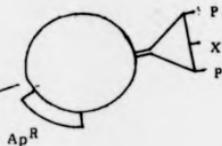
The 5.8kb Mx8att fragment was found to contain an internal Pat I site. To overcome this the Pat I bound 5.8kb Mx8att fragment was isolated from plasmid pPR107 by partial digestion using a 10⁻¹ dilution of enzyme over a time period of 0-10 minutes. The 5.8kb fragment was ligated into Pat I digested pDAH288. Transformants were selected on kanamycin and screened for ampicillin sensitivity. Insert size was determined by a Pat I digestion. A plasmid of the correct structure was identified and named pPR108. This plasmid was used to insert the Mx8att fragment into pDAH288, and pDAH287. The 18.58kb Hind III/Pat I fragments of pDAH288/287 were ligated to a 7.7kb Hind III partial Pat I fragment from pPR108. The 7.7kb fragment contains the entire 5.8kb Mx8att region and

Figure 24. Cloning scheme for the insertion of the 5.8 kb Mx8att fragment into plasmids pDAH286, pDAH286, and pDAH287. Plasmids pDAH286/287 and plasmids pPR110/111 differ in the orientation of the fragment containing the carQRS region. H) Hind III; P) Pst I; X) Xho I.

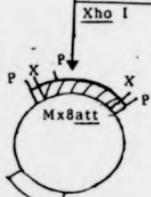
pMB500



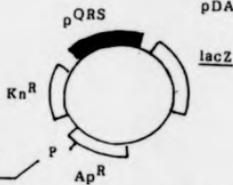
pMTL25(P)



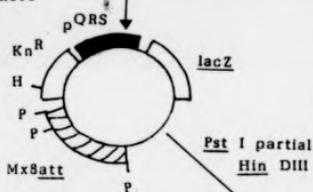
pPR107



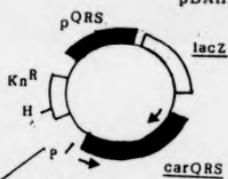
pDAH266



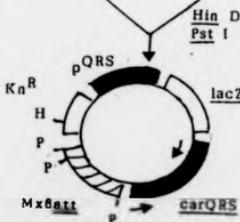
pPR108



pDAH286/287



pPR110/111



part of the kanamycin resistance gene cleaved from the 18.58kb fragment. This provides for the positive selection of chimeric molecules by the regeneration of a functional resistance gene. Insert orientation was determined by Xho I digestion. Plasmids of the correct structure were isolated and named pPR110, and pPR111. In pPR110 the p^{QRS} promoter is driving towards the kanamycin resistance gene, in pPR111 the orientation of the fragment is reversed.

4.2.3. p^{QRS} promoter expression from plasmid pPR108.

Plasmid pPR108 was P1 packaged and transduced into M. xanthus strain MR135. Transductants showing kanamycin resistance were picked in triplicate. To screen for possible spontaneous kanamycin resistance, and for the presence of plasmid sequence, colonies from two of the plates were hybridized to nitrocellulose. The colony lifts were probed with radiolabelled pDAH286, the parental plasmid for pPR108. All colonies showed the presence of sequences that hybridize to pDAH286.

M. xanthus MR135[pPR108] was patched onto DCY containing X-gal, a chromogenic substrate for beta-galactosidase. Plates were incubated in the light and the dark for 2-3 days. MR135[pPR108] showed a small amount synthesis of beta-galactosidase as measured by X-gal in the light and in the dark. Cells grown in the dark grew vigorously whereas cells grown in the light grew very poorly. The accumulation of carotenoids was not seen in either light or dark grown cells. The positive control for this experiment was strain M. xanthus DK101[pDAH217]. This strain showed high beta-galactosidase activity in the light compared to a small amount in the dark. Cells grew vigorously in the light and the dark, and carotenoids were seen

to accumulate in the light grown cells.

p^{QRS} activity from MR135[pPR108] was determined in liquid culture. p^{QRS} activity remained at between 2-4 units in the light and the dark. To determine if the lacZ was still functional, plasmid pPR108 was transformed into E. coli strain MC1061, in which promoter activity was determined as previously described (Chapter 3.1.2.1.). p^{QRS} activity from plasmid pPR108 was found to be comparable to p^{QRS} expression measured in previous experiments for strain MC1061[pDAH268]. The construction of pPR108 was repeated and transduced into MR135 to check for a constructional error leading to an artifactual result. No differences were found in the repeat experiment. p^{QRS} expression was monitored in M. xanthus DK101. In this strain promoter activity was found to be comparable to M. xanthus (DK101[pDAH217]). The plasmid pDAH268 was transduced into strain DK101. pDAH268 is identical to pPR108 except that it lacks the M_{6att} site and so will only integrate by homologous recombination with the carQRS region. The DK101 strain carrying the pDAH268 plasmid showed wild type p^{QRS} expression and wild type production of carotenoids.

4.2.4. p^{QRS} promoter expression from plasmids pPR110 and pPR111.

It was expected that in the absence of the carQRS region, i.e. in the absence of the repressor protein, expression from the p^{QRS} promoter would be at a high un-repressed level. p^{QRS} activity was found to be at a low level approximately equal to the levels of activity seen in a dark grown culture. This surprising result was investigated further to determine if full activity could be established by the introduction of the 5.6kb fragment of the carQRS region.

Plasmids pPR110 and pPR111 (see Figure 24. for details of plasmids) were P1 packaged and transduced into M. xanthus strain MR135. p^{QRS} expression from pPR110 was not detectable under light or dark conditions, giving values at zero or below. p^{QRS} expression from pPR111 increased three fold in the light rising from the level of a basal dark grown control at 8-9 Units, to a light induced level of around 25 units. To determine if homologous recombination had resulted in the deletion of the lacZ gene in M. xanthus (MR135[pPR110]) genomic DNA was isolated from this strain along with MR135 and MR135[pPR111] to act as controls, according to Methods 2.1.4.3.. Genomic DNA was digested with Pvu II and developed on a gel which was blotted onto nitrocellulose (Methods 2.1.5.). The filter was probed with the 1.5 kb Pvu II fragment of the lacZ gene which confirmed that the lacZ gene had been deleted in strain MR135[pPR110] (results not shown).

4.3. Discussion of results.

4.3.1. Light induction of p^{QRS} in M. xanthus.

Previous results Hodgson (1987) showed that light induction of the p^{QRS} promoter led to an increase in p^{QRS} activity to a peak value, which was followed by a decrease to a basal induced level. Results presented here have quantified the increase in carotenoids accumulating in light induced cells. It can be seen that the accumulation of carotenoids is concurrent with the decrease in p^{QRS} activity. An attempt was made to correlate the presence of carotenoids with removal of the inducer of p^{QRS} by addition of exogenous carotenoids but, commercially available carotenoids proved unsatisfactory. However, the reverse experiment was

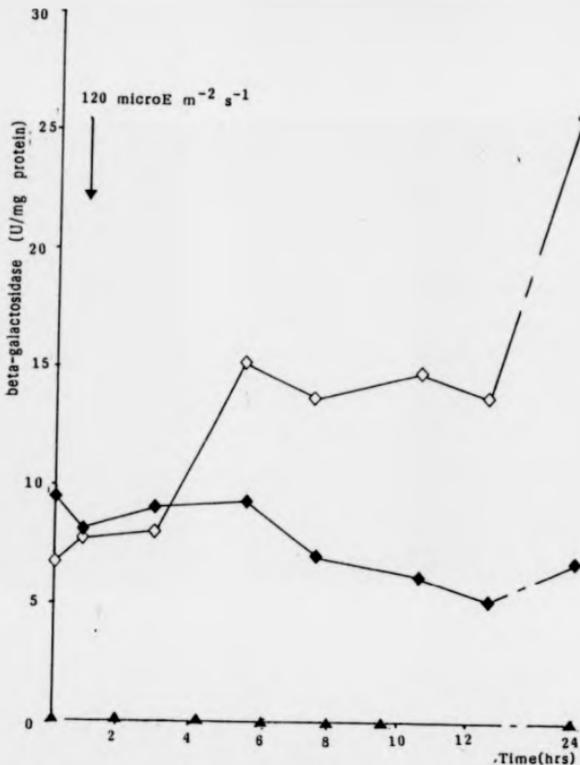


Figure 25. Expression of the p^{QRS} promoter inserted into the Mx8att site in a strain deleted for the entire carQRS region: \blacklozenge) p^{QRS} expression in the absence of the carQRS region; \blacktriangle) and \diamond) p^{QRS} expression in the presence of the 5.8 kb fragment containing the entire carQRS region, in either orientation.

successful, in that it was shown that in the absence of the full complement of endogenous carotenoids p^{QRS} activity does not show the characteristic decrease seen in experiments in which the host strain expresses a normal carotenoid response.

The induction of p^{QRS} under identical light intensities shows a dependency on growth phase. Cells from cultures in late exponential and lag phases show a decrease in the initial level of induction, as compared with cells from cultures earlier in the growth cycle.

The induction of p^{QRS} in cells from cultures at an identical growth phase, but by differing light intensity shows a direct proportionality between light intensity and p^{QRS} activity. This is interpreted as indicating that there is a single photoreceptor responsible for the induction of p^{QRS} between 35-550 $\mu\text{m}^{-2} \text{s}^{-1}$. However, it should be noted that other photoreceptors may contribute to the induction but at a sufficiently low extinction coefficient that their effects are not seen in this experiment. Previously Burchard and Hendricks (1988) used spots of cells on agar plates exposed to differing light intensities to extract carotenoids. They showed that there was direct proportionality between light intensity and carotenoid accumulation until a saturation point is approached. Under the culture conditions used in this study the saturation point, if one exists, was not reached.

It has been shown that laser light of defined wavelength at 410nm will cause a large increase in p^{QRS} activity. The intensity is irrelevant to interpretation since if the photoreceptor was unable to absorb a particular quanta of light the flux of the light would have no effect on the receptor. This wavelength corresponds to the maximum absorption (Soret) peak seen in all porphyrins. However, both carotenoids and flavins also absorb in the

blue region. The effect of additional wavelengths on the induction of p^{QRS} needs to be studied, particularly those above 520nm which cannot be absorbed by carotenoids or flavins.

It is interesting to note that the intensity of defined wavelength light required to produce a detectable increase in p^{QRS} activity appears to be inordinately high when compared to the intensity of white light routinely used to induce the promoter to high levels of expression. Filtered light at an intensity of $22 \text{ microE m}^{-2} \text{ s}^{-1}$ had no detectable effect on promoter activity, and laser light of $40 \text{ microE m}^{-2} \text{ s}^{-1}$ had only a small effect, increasing activity from 3-5 units. This increase may not be significant since the level of activity in dark grown batch cultured cells shows a similar rise. The result obtained using laser light may be explained in that laser light is provided in pulses, and it is possible that pulses of high intensity light are not as effective at inducing the response as is continuous low intensity light. However, the filters used an experimental design identical to that used in routine white light inductions, except, a higher intensity of white light was provided as a source. This result suggests the possibility that other wavelengths, either, the submaxima of the protoporphyrin IX absorption spectrum, or quanta absorbed by a different molecule, are of importance in the induction of the p^{QRS} promoter.

4.3.2. The use of the bacteriophage Mx8att site as an alternative integration site.

Using the Mx8att fragment a construct containing the p^{QRS} promoter driving through a promoter-less lacZ gene was introduced into a strain

deleted for the entire carQRS region. It was expected that in the absence of the carR gene product, the repressor, the promoter would be de-repressed i.e. constitutively expressed. However, this strain showed very low p^{QRS} activity, as measured by X-gal and ONPG. p^{QRS} activity was not inducible by light which, as expected, inhibited growth. The construct showed normal levels of expression in E. coli, and exhibited wild-type expression in M. xanthus strain DK101. This suggests that there is an additional factor required for the expression of the promoter which is not present in strain MR135, and is not present on the pPR106 plasmid.

Introduction of the 5.8kb fragment of the carQRS region imparted not only the ability to synthesise carotenoids, when exposed to light; but also rendered the p^{QRS} promoter, upstream to the lacZ reporter gene, light inducible. The promoter does not show the very high levels of expression seen when the promoter is integrated by homologous recombination into the carQRS region. This is most likely a consequence of integration into the att site. It has been shown that promoters integrated into this site very often show a reduced activity (R.Gill and L.Kroos pers. comm.).

Previous results had shown that carR and carS were not required for the stimulation of p^{QRS} activity (D.Hodgson, pers. comm.). Mutation in the carR gene resulted in high levels of p^{QRS} activity and a Car^C phenotype. A strain containing an inactive carS gene, which is unable to synthesise carotenoids, still shows light induction of the p^{QRS} promoter, thus illustrating that the product of the carS gene does not activate the p^{QRS} promoter. Therefore it is suggested that some other protein expressed by the 5.8kb carQRS region, most probably the carQ gene product, is responsible for the high levels of promoter activity seen after light induction. This is currently being investigated in D.Hodgson's laboratory.

Initial results have confirmed this proposal in that a construct containing the p^{QRS} promoter, extended at the 3' end to encompass the entire carQ gene, in the absence of both carB, and carS, showed a constitutive high level of promoter activity (S.McGowan pers. comm.).

CHAPTER 5. The effect of light and oxygen on promoter activity in M.xanthus (DK101[pDAH217]) under conditions of continuous culture.

5.A.1. The theory of continuous culture.

There are numerous texts published regarding the theory and practice of continuous culture (Dykhuizen and Hartl, 1983; Harder et al., 1977; Tempest (1970)). As such only a general introduction shall be considered here. The theory of continuous culture may best be explained with reference to mathematical models of the growth of a microorganism in culture. Such models were first proposed by Monod (1949) who noted that when inoculated into suitable media, microorganisms will grow at a rate which is the maximum possible under the given conditions. During growth in batch culture the environment of the microbe in a growing culture will change, but growth will continue at a maximal rate until the change is such to prevent this, eg. depletion of an essential nutrient. If all nutrients are in excess the substance which limits growth is called the limiting nutrient. The growth rate of a microorganism is dependent on the concentration of the limiting nutrient. Such growth in batch cultures may be represented by the Monod equation:

$$\mu = \mu_{max} \cdot s / K_s + s$$

where μ is the specific growth rate, μ_{max} is the maximum specific growth rate, s is the concentration of the growth limiting substrate, and K_s is a constant numerically equal to the substrate concentration at which μ is $1/2 \mu_{max}$.

In a continuous culture a micro-organism is continuously supplied with fresh medium which is immediately mixed with the culture, excess medium is displaced from the culture such that the volume remains constant. This allows the microbial population to be examined under conditions in which they do not constantly perturb the environment, and in which a steady state is achieved, whereby all measurable parameters, including growth rate, remain constant. The two most common forms of continuous culture are the chemostat and the turbidostat. Only the chemostat shall be discussed here.

In a chemostat the growth rate is determined by the dilution rate of the culture (D), where $D=f/V$; where f = volume of fresh media added per unit time, and V = volume of the culture (a constant). The change in the concentration of micro-organisms with time (dx/dt) will be dependent on the growth rate (μx) and the dilution rate (Dx), where x is the dry weight of micro-organism; so:

$$dx/dt = \mu x - Dx \text{ or, } dx/dt = x(\mu - D)$$

It can be seen that only when $\mu = D$ will the concentration of microorganisms remain constant with time, this is the steady state. If μ exceeds D the concentration of micro-organisms will increase. The reverse is true if D exceeds μ . When μ exceeds D , the consumption of substrate will be larger than the input, and the substrate level will gradually decrease; consequently according to the Monod equation μ will decrease until $\mu = D$ is achieved. This shows, and has been confirmed in numerous additional analyses, that providing that D does not exceed the critical dilution rate then a steady state where $\mu = D$ will be achieved. The critical dilution rate is invariably the equivalent of the maximum growth

rate of the micro-organism in the particular medium used (Harder et al 1977).

5.A.2. The application of continuous culture to the study of microbial physiology.

The limitations of growth in batch culture for the study of microbial physiology have already been alluded to and shall not be reiterated here. The main advantage of studying a population of cells under continuous culture steady state, is that the cells are not constantly changing and adapting to a changing environment. Thus their physiology remains comparatively constant. This enables the study of the effect of changing a particular parameter, while keeping constant others which may exert an influence. In this study, continuous culture has allowed the effect of variation of illumination, and/or variation in oxygen tension, to be examined in isolation from other possible parameters such as growth phase, and pH (Chapter 4.1.4.; Hooper and Phinney (1988(a))).

Some examples of the use of continuous culture to examine the effects of light on micro-organisms may be found in the following papers; Aiking and Sojka (1979) described the effects of illumination and growth rate on cell composition and pigment synthesis in Rb. capsulata; Steinborn and Oelze (1989) studied photosynthetic activities under different illuminations in the same organism. Dingler and Oelze (1987) have reported the effects of variation in the oxygen tension, of a continuous culture of Azotobacter vinelandii, on the levels of catalase and superoxide dismutase. The subject of these papers does not warrant further description here.

5.1. Design of chemostat.

The continuous culture device used was an LHE 1/1000. It consisted of a glass and steel 3 litre chamber, with ports for:- pH electrode, oxygen electrode, thermocouple, heating element, media addition, acid addition, alkali addition, air addition, air outlet, culture overflow outlet, sampling outlet, and cold finger. The culture was baffled, and agitated at 4000 r.p.m. by means of a paddle.

Preliminary batch experiments determined that growth of M. xanthus in complex media resulted in an increase in pH from 7.8 to 8.8. This is in agreement with Gerth et al. (1984), who noted that when myxobacteria are grown on protein and peptide rich media the pH increases, this increase was attributed to the release of ammonia. In chemostat studies the culture pH was maintained at 7.8+/-0.2 by the addition of 0.5M hydrochloric acid.

Preliminary chemostat studies had identified three problems in growing M. xanthus which were due to the cells adhesiveness. Firstly the media-in tube can be colonized and growth may spread into the sterile media reservoir. This was prevented by inserting an anti-growth-back tube close to the chamber. This tube breaks the flow of liquid through which cells may swarm by inserting an enclosed air-space through which media is dropped. Secondly any rough surface may be colonized by M. xanthus. This was prevented by silconising all glass within the chamber, which dissolves and smoothes the surface preventing adherence. Thirdly the enclosed anti-splash guard used in most overflow devices was easily colonized and was replaced by a length of glass tubing.

Aeriation was controlled by two meters. A main air intake, and a secondary intake which may be provided by compressed gas. Both Ingressive

and egressive air was passed through a filter. When oxygen was used, spent gasses were conveyed directly to the atmosphere. Oxygen tension within the fermenter was measured by means of a Russell combination O_2 electrode type G2. The metered values were established by aerating to equilibrium, routinely for 2 hours, the fermenter with pure nitrogen, for the 0% value; and with pure oxygen, for the 100% value. To avoid variation in mass transfer of gasses to the culture, the air flow to the chemostat was kept constant by varying the air and compressed gas (N_2 or O_2) accordingly.

The heating effect due to the light sources was minimised, firstly by circulating cold water in a steel finger inside the chamber, and secondly by maintaining a flow of air around the chamber by means of a small fan. Culture temperature never varied by more than $\pm 0.5^\circ C$.

Regular measurements were taken to ensure steady state conditions were maintained. The following parameters were monitored: pH, temperature, air and media flow rates, agitation rate, and culture optical density. Samples were withdrawn for assay of beta-galactosidase, and carotenoid content, when required, and processed according to Methods.

5.2. Use of defined medium (A1) in continuous culture.

Defined medium (A1) is described in Methods 2.2.4.. This was chosen for three reasons. Firstly, the tendency of M. xanthus to lyse in rich media is reduced. Secondly, unlike DCY, it will not foam upon rapid agitation. Thirdly, it is relatively inexpensive.

M. xanthus was batch cultured in A1 medium to determine its growth parameters in this medium. The doubling time during exponential growth

was determined as 38 hours, giving a maximum growth rate of 0.0178 hr^{-1} . The culture attained a final O.D.₆₈₀ of around 0.25.

100ml batch cultures grown in DCY and in A1 media were used as inocula for the chemostat. A1 medium was added at 50ml/hr. giving a doubling time of 50 hours.

Growth in A1 medium alone gave low optical densities at around 0.16. Addition of DCY. to a continuous culture increased the OD₆₈₀ but this decreased again on dilution with A1 medium.

The use of A1 medium was abandoned for two reasons. Firstly, low optical density necessitated harvesting large volumes of cells for assay, which significantly altered dilution rates. Secondly, long doubling times required long transition times before a steady state could be said to have been achieved, this was not practical for this study.

5.3. Use of complex media in continuous culture.

The use of complex media has several drawbacks. It is more expensive, cultures are prone to foaming, and the limiting nutrient is difficult to determine. When using complex media the nature of the limiting factor would probably vary with parameters such as temperature and media flow rate (Tampest, 1970). The complex medium DCY (Methods 2.2.1.4.) is a commonly used complex media for the cultivation of *Mycococcus* species, half strength DCY. (DCY(H).) was used in this study to reduce expense.

The following antifoam agents were tested for a possible inhibitory effect on *M. xanthus*: Pluron (0.01%), anti-foam A (0.1%), Polypropylene glycol (PPG.) (0.015%), and anti-mousse 420R (150 micro-g/ml). Anti-

mousse 428R and PPG showed no inhibitory effect. PPG at 0.015% was used in this study and was found to adequately prevent foaming. The limiting nutrient could not be determined by the addition of casamino acids, yeast extract, pyruvate, magnesium sulphate, or casitone to a continuous culture of M. xanthus in DCY(H). However, the OD_{660} was seen to decrease by 0.3 Unit on addition of casitone (2% w/v DCY(H)). Dworkin (1962) had shown that certain amino acids at higher concentrations could be toxic, causing an imbalance or antagonism which presumably inhibited the entry of other, essential amino acids into the cell. This suggests that addition of complex supplements while providing the limiting factor, may also provide inhibitors of growth thus masking their effect.

All subsequent chemostat studies employ DCY(H).

5.4. Steady state growth using complex media.

2.5 litres of DCY(H) in the chemostat was inoculated with 100mls of a lag phase, overnight culture of M. xanthus DK101[pDAH217] with an optical density OD_{660} between 1.5-1.7. This culture was allowed to grow to mid-exponential phase ($OD_{660}=0.8-1.3$), after which media addition to give a growth rate of 0.035 hr^{-1} , ($t_d=20\text{hrs.}$), was begun. The culture was maintained until all measurable parameters: optical density, pH, and oxygen tension, remained constant for 5 volume changes; at which point a steady state was said to have been achieved. See Figure 24 for growth parameters. The maintenance of the plasmid, pDAH217, was selected for by the presence of kanamycin at 50 micro-g/ml in all media. A number of checks were performed regularly on the chemostat during operation. 1) The pH metered at the chemostat was checked against an external standard. 2)

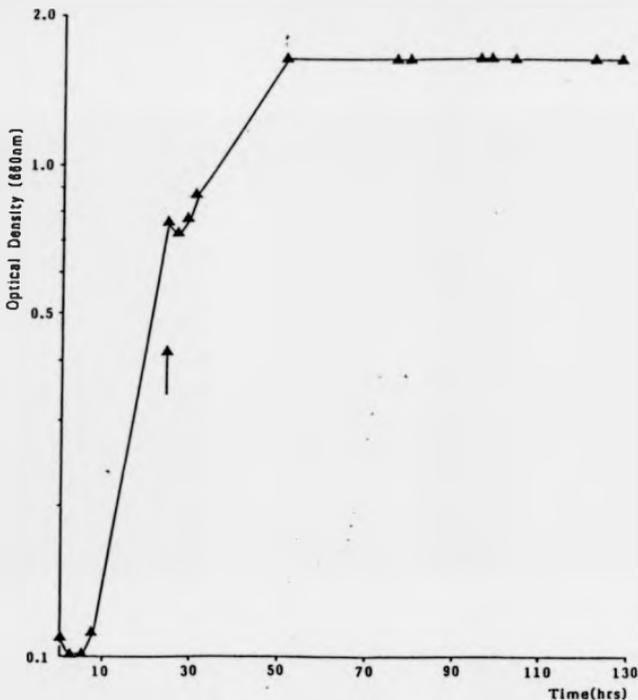


Figure 26. Growth curve of a culture of *M. xanthus* maintained in a chemostat: 2.5 litres of DCY(H) was inoculated with an over-night culture of *M. xanthus* and grown to mid-exponential phase at which point dilution with fresh medium was begun (shown by arrow). A steady state growth rate of 0.035hr^{-1} is shown. \blacktriangle) A 660.

The media flow rate was checked by means of a pipette attached to the media-in tube, as variation may occur when the tubing becomes stretched after continual use. 3) Culture purity was assessed in withdrawn samples by phase contrast microscopy and by cultivation on solid media. If contamination was detected experiments were abandoned.

Due to the projected length of some chemostat experiments it was decided to crudely test for adaptation to continuous liquid growth. This was done in two ways. Firstly, by comparing the batch culture growth rate of a chemostat sample against the batch growth rate of a plate stock. Secondly retention of the ability to sporulate was tested on T.M. media, which consists of tris/magnesium solidified by 1.5% agar on which M. xanthus cannot grow but which promotes sporulation. Sporulation requires the presence of pili. Pili lead to "stickiness" of cells, as such continuous culture actively selects for the loss of pili in that fragmentation will prevent wash-out occurring. No differences between plate stocks and continuous liquid culture were detected by either method in any of the following experiments. As a rule the accumulation of mutants in continuous culture should not be significant if experiments are completed within 1000 volume changes (Tempest 1976). To adhere to this "rule of thumb" two experiments were not run contiguously and fresh inocula were prepared from plate stocks and not from the culture in the chemostat. The above protocols were employed in the routine running of all subsequent continuous cultures described hereafter, unless otherwise stated.

5.5. The study of how oxygen and light effects p^{QRS} expression in M. xanthus under continuous culture with a slow growth rate.

5.5.1. Variation in oxygen tension only.

The chemostat was maintained in dark conditions, a steady state was achieved with an optical density between 1.6-1.8. Oxygen tension was decreased to between 25%-35% at which a steady state was achieved. The 100% value for aeration was set at the start of the experiment (see section 5.1.). Aeration was increased over 20 minutes, to between 75%-85%. The air flow rate was maintained at 350 ml/min. Phase variation of colony colour from yellow to tan increased on increasing aeration. Promoter activity was determined and rose approximately two fold (Figure 27). Variations in promoter activity required that statistical analysis be carried out on this data to determine if the observed increase was due to increasing aeration, or was due to random scatter. Students T-test was performed, which showed a significant increase in promoter activity occurred after the time at which oxygen tension was increased.

5.5.2. The effect of light at high and low oxygen tensions.

A statistically significant increase in p^{QRS} expression has been shown to occur after increasing the aeration of a dark grown culture. The increase was not as marked as was anticipated, however, the possibility arises that p^{QRS} induction in a steady state culture will not be to the levels seen in batch culture. To determine if this is so, and to study the effect of oxygen tension on the level of p^{QRS} induction, the effect of identical light treatments on steady state cultures of M. xanthus

Figure 27. The effect of increasing the oxygen tension on p_{QRS} activity in cells of a dark grown, continuous culture, of M. xanthus with a growth rate of 0.035 hr^{-1} ; Δ) p_{QRS} activity as measured by beta-galactosidase activity, \bullet) oxygen tension.

Figure 27.

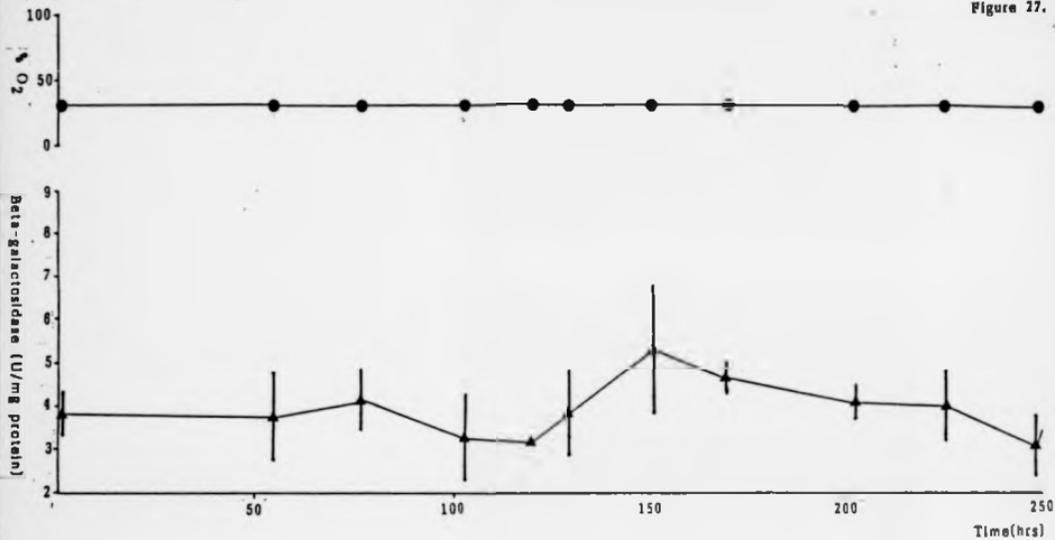
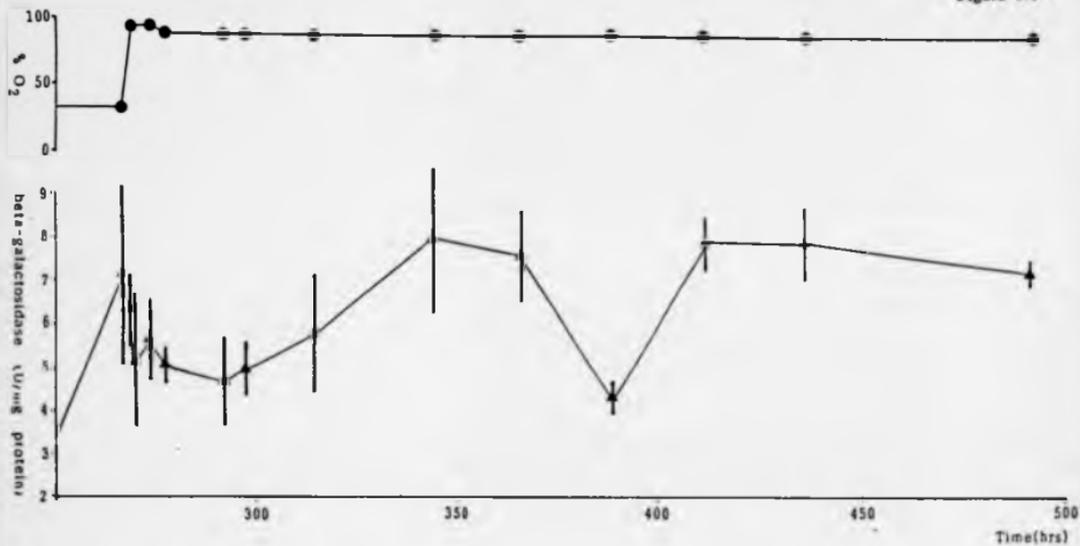


Figure 27.



maintained at high and low oxygen tensions was studied.

The chemostat was maintained in dark conditions, described previously, and a steady state was achieved with an optical density between 1.85-1.85. The culture of 2500ml was diluted with fresh media at a rate of 115ml/hr to give a growth rate of 0.035 hr^{-1} which represents a doubling time of 20 hr. Promoter activity was examined under six steady states in sequence. Light values given are in micro-Einsteins $\text{m}^{-2}\text{s}^{-1}$. The sequence of states was as follows:- 1) Dark/High oxygen tension (80-90%), 2) Low light intensity (300)/High oxygen tension (80-90%), 3) High light intensity (1500)/High oxygen tension (80-90%), 4) Dark/Low oxygen tension (1-4%), 5) Low light intensity (300)/Low oxygen tension (1-4%), 6) High light intensity (1500)/Low oxygen tension (1-4%). A stepped increase in light intensity was employed to guard against photolysis due to high light intensity. A distinction in pigmentation was seen in that the samples grown under high oxygenation plus high light were orange/red, whereas samples grown under low oxygenation plus high light were orange. Low light caused little observable increase in pigmentation.

Expression from p^{QRS} was monitored and is shown in Figure 28, along with the measured oxygen tension and the points at which light treatment was altered. Promoter activity under high oxygen tensions increased two-fold from a basal level of 6-7 units to 10-15 units under low and high light intensity. Under low oxygen tensions low light intensity gives a similar increase in activity, high light intensity gives a 2-3 fold increase to 18-25 Units. One rogue value of 180 units is noted at 957hr., this corresponds to an increase in oxygen tension from 1% to 20% caused by the loss of nitrogen addition to the fermenter. Consequently steady state was lost and not regained until around 1130 hrs.

Figure 28. The effect of high and low oxygen tensions on the level of induction of p_{ORS} by light in cells of a continuous culture of M. xanthus, with a growth rate of 0.035 hr^{-1} : The points at which the culture was exposed to light are marked by arrows, which also indicate the light intensity in $\text{microE m}^{-2} \text{ s}^{-1}$. \blacktriangle) p_{ORS} activity as measured by beta-galactosidase activity, \odot) oxygen tension.

Figure 28.

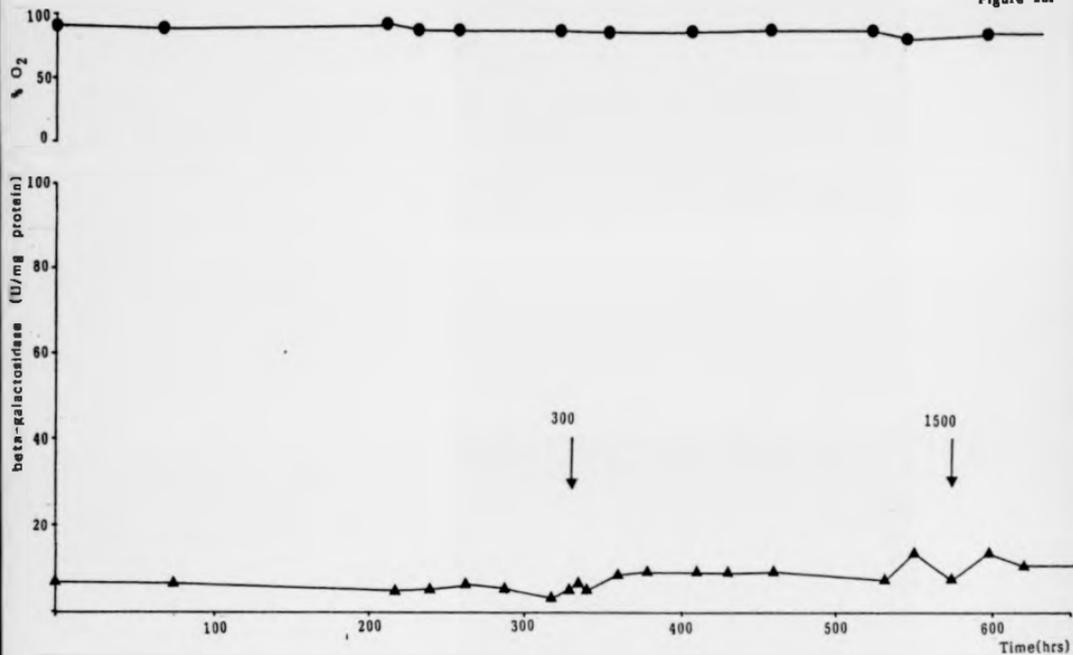
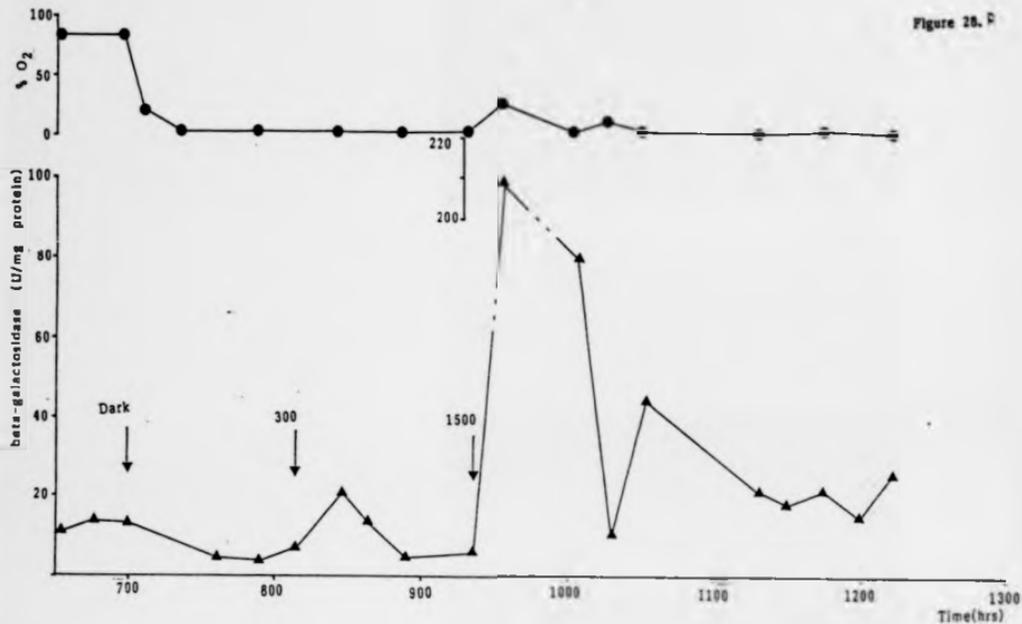


Figure 28. F



5.8. The study of how oxygen and light affect p^{QRS} expression in M. xanthus grown under continuous culture with a faster growth rate.

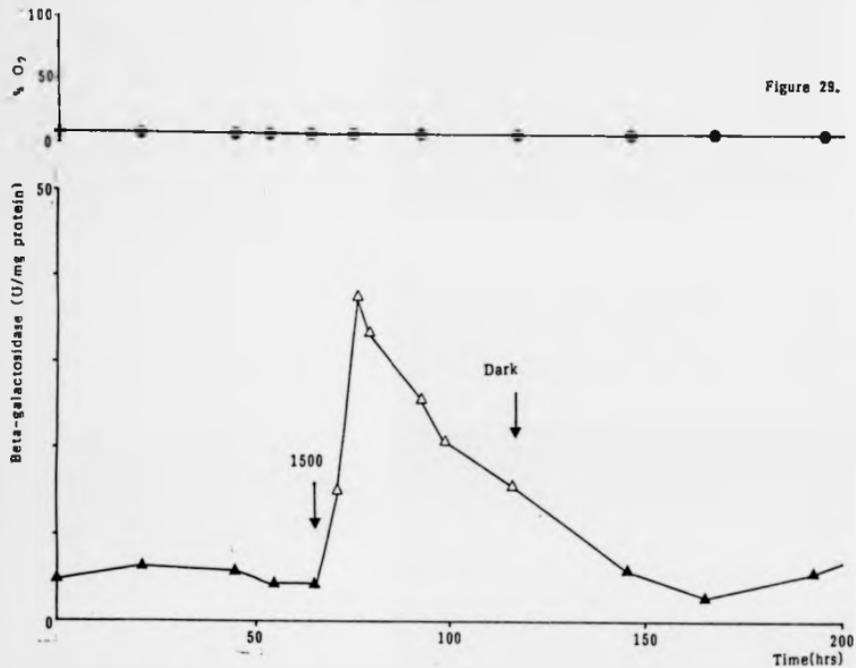
Due to the apparent lack of detectable p^{QRS} activity in continuous cultures with a growth rate of 0.035 hr^{-1} ($t_d=20\text{hr}$). It was decided to increase the growth rate. This had two advantages. Firstly according to previous results, (Chapter 4.3.) in response to identical light stimuli promoter activity decreased as a culture progressed through the growth curve. It is possible that this result may be applicable to a continuous culture, however, the two conditions of culture may not be comparable. Secondly, steady state conditions will be achieved quicker allowing more rapid attainment of data and reducing the likelihood of equipment failure over protracted time courses. The fermenter volume was decreased to 1500ml and the media flow rate was determined at 150 ml/hr. This gives the culture a growth rate of 0.069 hr^{-1} ($t_d=10\text{hr}$). Attempts to get the growth rate higher than 0.069 hr^{-1} , towards the maximum growth rate in batch culture of 0.2 hr^{-1} , resulted in wash out of the culture. A second improvement in experimental design was employed. The stepped exposure to light was abolished in favour a single exposure to light at 1500 $\mu\text{E m}^{-2} \text{ s}^{-1}$. This was to allow for the possibility that low light levels, allow a low induced level of carotenoid synthesis to be established, which is sufficient to mask the effect of the high intensity light. It would be expected that such an effect would be most pronounced in a culture in which cells have a long doubling time in which despite a low level of p^{QRS} induction high levels of carotenoid may accumulate.

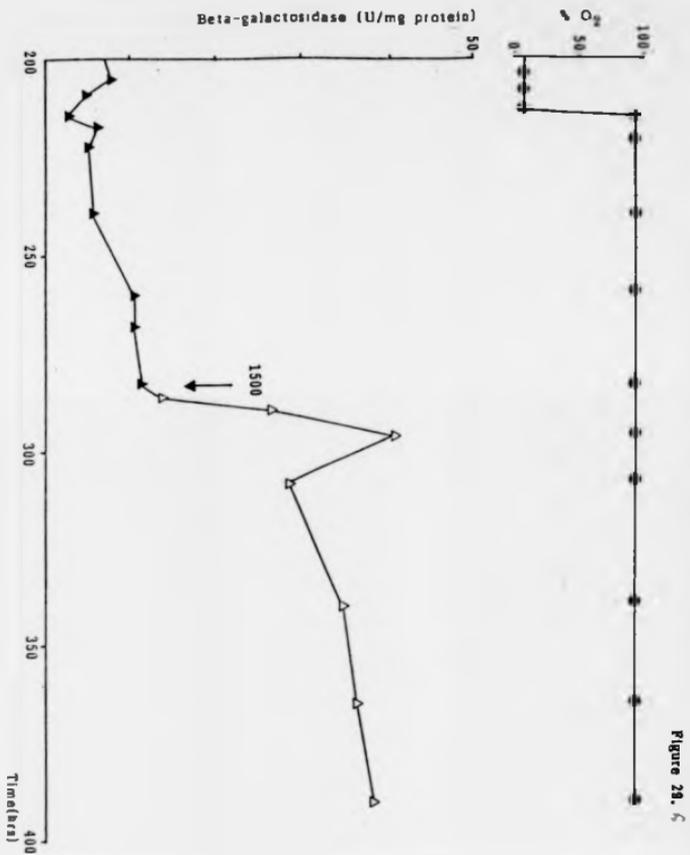
5.8.1. Control test for p^{QRS} activity at high and low oxygen tensions in the light and in the dark.

A test run was performed to ensure that the p^{QRS} promoter would express at detectable levels under high and low oxygen tensions. 1.5 L of media was inoculated with a 100ml lag phase overnight batch culture of M. xanthus DK101[pDAH217] at an OD_{680} of 1.5-1.7. This was cultivated under high oxygen tension until a steady state was achieved with an OD_{680} of 1.8-1.8. The oxygen tension was reduced to 17.5% and a steady state achieved with an identical OD_{680} , at this point experimental assays were commenced. Promoter activity was measured in sequence as follows: 1) Dark at low oxygen tension (17.5%); 2) Light at low oxygen tension; 3) Dark at high oxygen tension (80%); 4) Light at high oxygen tension. Light levels were measured at $1500 \text{ microE m}^{-2} \text{ s}^{-1}$. Low oxygen tensions were achieved by passing oxygen free nitrogen through a culture in addition to a lower level of aeration. Under high oxygen levels the nitrogen was replaced by pure oxygen.

p^{QRS} activities were assayed and are shown in Figure 29. p^{QRS} activity increased to 39 Units after low oxygen tension grown cells were exposed to light. The promoter activity decreased over the next 50hr to a value of around 15 Units, at which the culture was returned to dark conditions. Increased oxygenation caused a slight increase, around 2 fold, in p^{QRS} activity. The light stimulus caused an increase in activity to 42 Units. Subsequently p^{QRS} activity was maintained between 35-40 Units over the next 100hr, after which the experiment was terminated.

Figure 29. The effect of high and low oxygen tensions on the level of induction of p^{QRS} by light in cells of a continuous culture of M. xanthus, with a growth rate of 0.069 hr^{-1} : The points at which the culture was exposed to light are marked by arrows, which also indicate the light intensity in $\text{microE m}^{-2} \text{ s}^{-1}$. Δ) p^{QRS} activity as measured by beta-galactosidase activity, \bullet) oxygen tension.





5.8.2. p^{QRS} activity at low and high oxygen tensions in the dark.

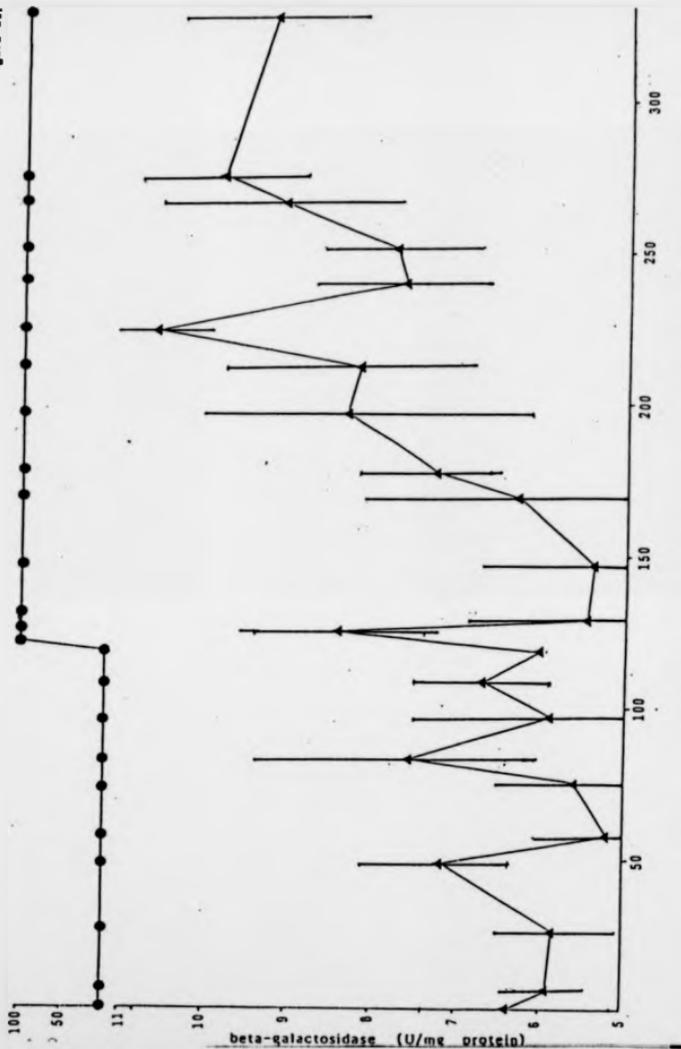
Having established that high levels of p^{QRS} activity may be measured from cells in continuous culture with a growth rate of 0.069hr^{-1} , a similar experiment to that described in section 5.5.1. was performed. A steady state dark grown continuous culture was achieved as previously described in Section 5.6.1. with an OD_{880} between 1.8-1.8. This culture was maintained in dark conditions previously described. The oxygen tension was decreased to 15-18% and a steady state achieved. p^{QRS} activity was assayed and is shown in Figure 30., under low oxygen tension and after a subsequent shift to enhanced oxygen tension (100%). Dark conditions were maintained throughout. Under low oxygen tension p^{QRS} activity varied between 5.4 and 8.4 Units. After the increase in oxygen tension p^{QRS} activity varied between 5.4-10.0 Units. It was noted that the increase in oxygen tension caused a decrease in the scatter of values for p^{QRS} activity. Students T-test confirmed that there is a significant increase in promoter activity after oxygenation of the culture is increased.

5.8.3. p^{QRS} activity at high and low oxygen tensions in the light and the dark with extended steady states.

The control experiment described in section 5.6.1. indicated that one of the effects of increased oxygen tension on the expression of p^{QRS} after induction by light was to maintain a higher level of expression after the initial peak. In addition it was noted that in dark grown cultures the scatter of values is greater under low oxygen tension than it is under high oxygen tension. To determine the significance of both these observed

Figure 30. The effect of increasing the oxygen tension on p^{QRS} activity in cells of a dark grown, continuous culture, of M. xanthus with a growth rate of 0.089hr^{-1} : Δ) p^{QRS} activity as measured by beta-galactosidase activity, \bullet) oxygen tension.

Figure 30.



phenomena, light induction of p^{QRS} activity in cells continuously cultured at various oxygen tensions was performed, allowing long steady states to be achieved. Additionally it was expected that if a culture were to be maintained under limiting oxygen tension, whereby oxygen is the limiting nutrient and determines the optical density to which the culture will grow, the level of p^{QRS} induction by light should be minimal if present at all. The effect of limiting oxygen tension is reported below. A steady state culture was achieved as previously described in section 5.8.1. at an OD_{660} of 1.8-1.8. The oxygen tension was decreased to 1-2% as previously described, using nitrogen to balance the mass transfer of gasses and to lower the dissolved oxygen tension further. At this oxygen tension it was noted that the OD_{660} decreased to 1.2-1.4, the most likely explanation for this phenomena was that oxygen has become the limiting nutrient; this was later confirmed when increasing oxygenation resulted in an increase in the OD_{660} to 1.8-1.8. Promoter activity was measured in sequence as follows: 1) Limiting oxygen (1-2%) in the dark; 2) Limiting oxygen (1-2%) in the light; 3) High oxygen (80-85%) in the dark; 4) High oxygen (80-85%) in the light; 5) Enhanced oxygenation (100%) in the dark; 6) Enhanced oxygenation (100%) in the light.

p^{QRS} activity was assayed and is shown in Figure 31. Under limiting oxygen tension p^{QRS} activity in the dark varied between 1-3 Units. On exposure to light promoter activity increased to 32 Units over 22 hours and steadily declined over 120 hours to 7 Units. At this point the culture was returned to dark conditions and a steady state culture achieved at a higher oxygen tension measured at 70-80%. Promoter activity in the dark was 4.5-8.5 Units. On exposure to light promoter activity increased to 55 Units over 22 hours and was maintained at between 48.5-50 Units over the

Figure 31. The effect of enhanced, high, and limiting oxygen tensions on the level of induction of p^{QRS} by light in cells of a continuous culture of M. xanthus, with a growth rate of 0.008hr^{-1} : The points at which the culture was exposed to light are marked by arrows, which also indicate the light intensity in $\text{microE m}^{-2} \text{s}^{-1}$, Δ) p^{QRS} activity as measured by beta-galactosidase activity, \odot) oxygen tension.

Figure 31.

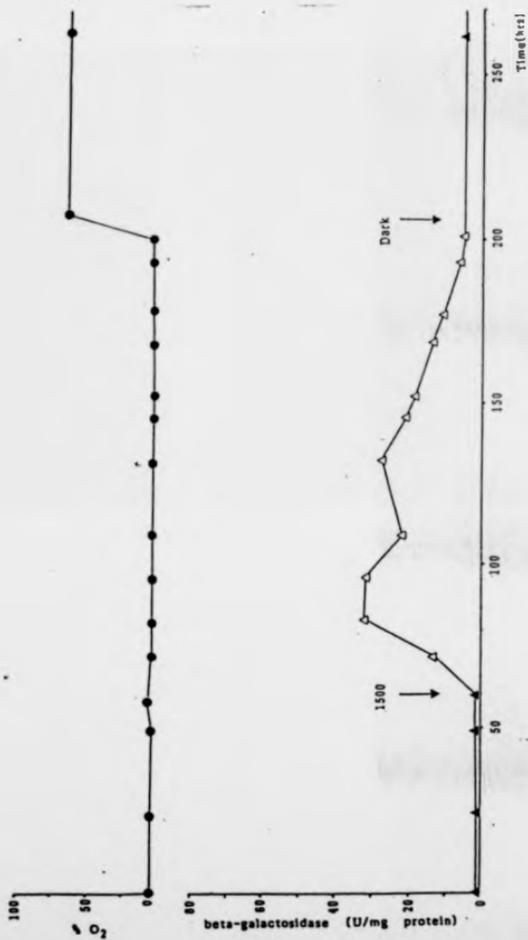


Figure 31.9

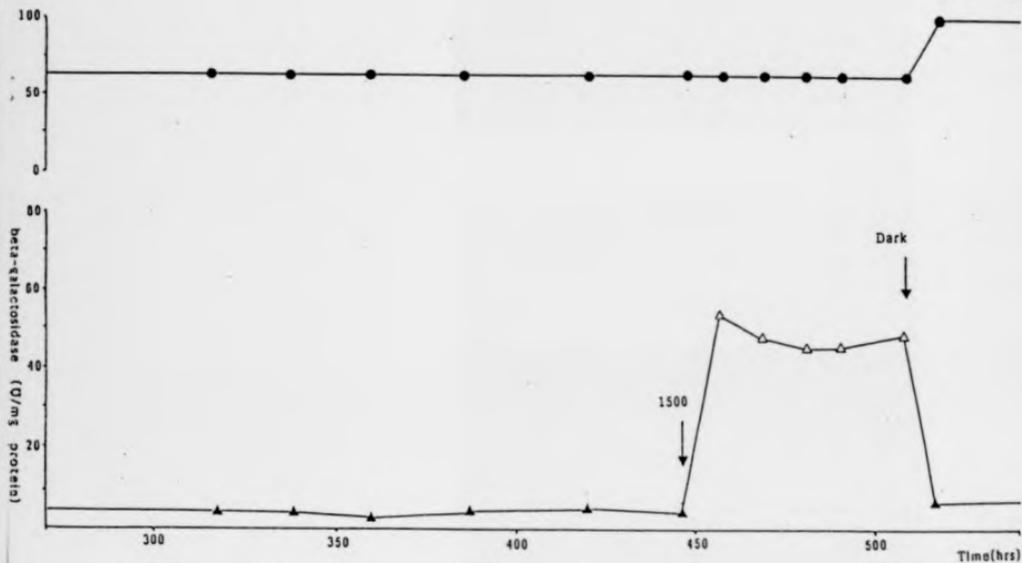


Figure 31.4

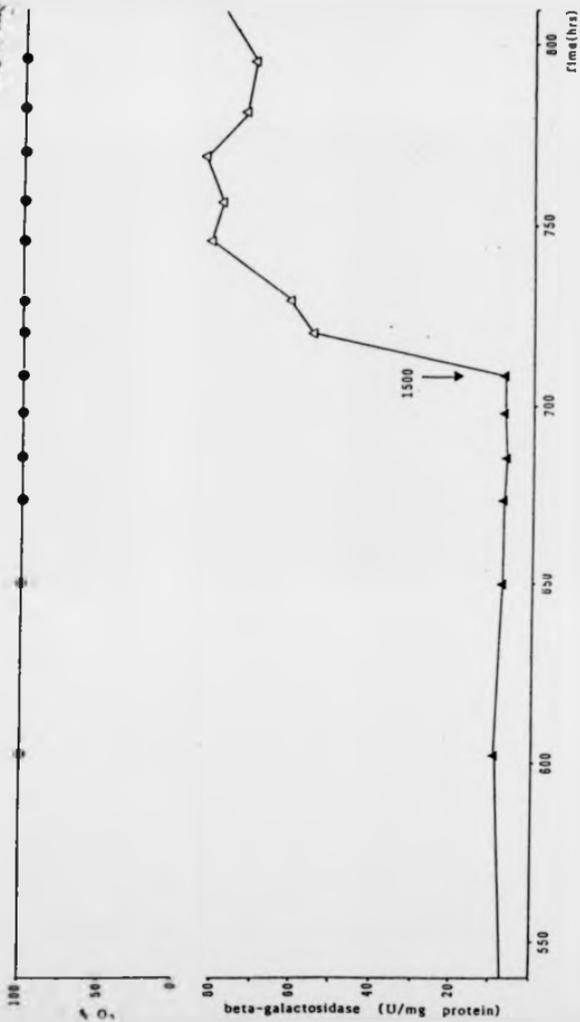
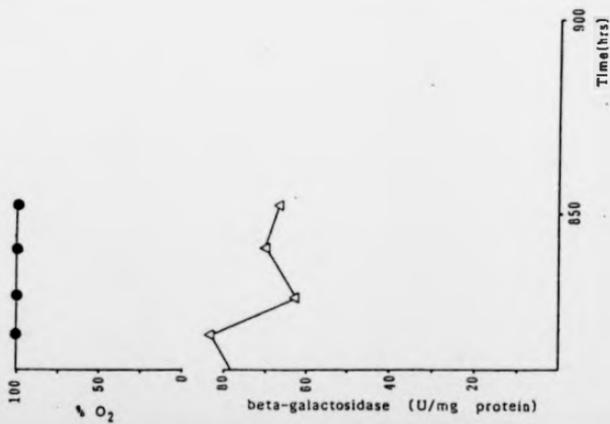


Figure 31.5



next 85 hours. At this point the culture was returned to dark conditions and oxygenated with pure oxygen. This increased the oxygen tension to 100% and a steady state was maintained. Promoter activity in the dark was 7-9.5 Units. On exposure to light promoter activity increased to 83 Units over the next 25 hours and was maintained at between 85-83 Units over the next 105 hours. At which point the experiment was terminated.

5.6.4. p^{QRS} activity of a light induced culture shifting from low to high to hyperoxygenated tensions.

It had been noticed in previous experiments that the pigmentation of pelleted cells was dependent on the level of aeration of the continuous culture. In addition if oxygen is the determining factor in the level of induction seen in a continuous culture it may be expected that a light induced culture of M. xanthus may be further induced by the addition of oxygen. This was addressed by studying pigmentation and p^{QRS} activity in a culture, which was light induced at low oxygen tension, then exposed to increasing levels of aeration. A steady state continuous culture was achieved as previously described in Section 5.6.1. with an OD_{880} between 1.5-1.7. The oxygen tension of this culture was reduced to between 4-8%, and a new steady state achieved, with an OD_{880} of 1.5-1.7. Promoter activity was assayed under the following sequence of culture conditions: 1) Dark and low oxygen tension (4-8%); 2) Light and low oxygen tension (4-8%); 3) Light and high oxygen tension (80-85%); 4) Light and elevated oxygen tension (100%).

p^{QRS} activity data are shown in Figure 32. Under dark conditions p^{QRS} activity varied between 5-7 Units. On exposure to light this value

Figure 32. The effect of increasing the oxygen tension on p^{QRS} activity in cells of a continuous, light grown, culture of M. xanthus with a growth rate of 0.089hr^{-1} : The point at which the culture was exposed to light is marked by an arrow, which also indicates the light intensity in $\text{microE m}^{-2} \text{s}^{-1}$. Δ) p^{QRS} activity as measured by beta-galactosidase activity, \bullet) oxygen tension.

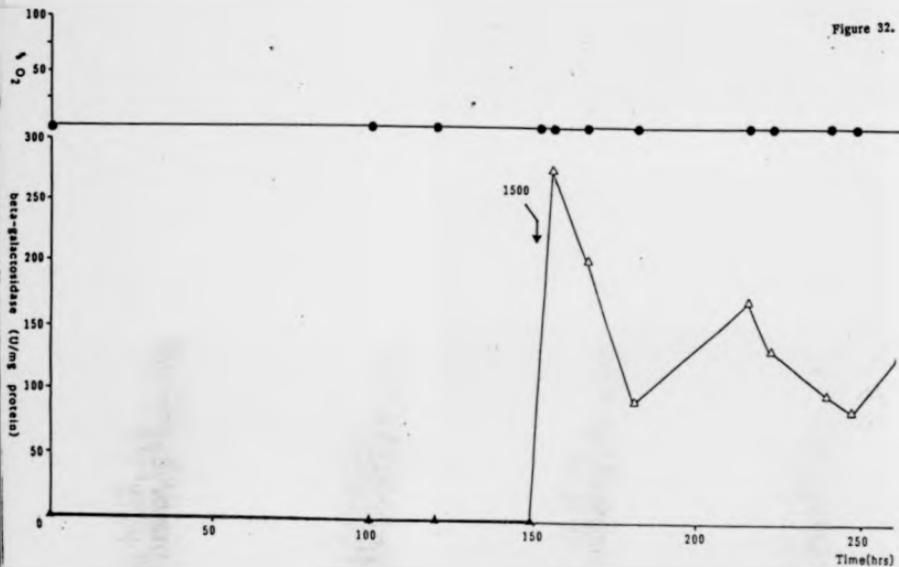


Figure 32. F

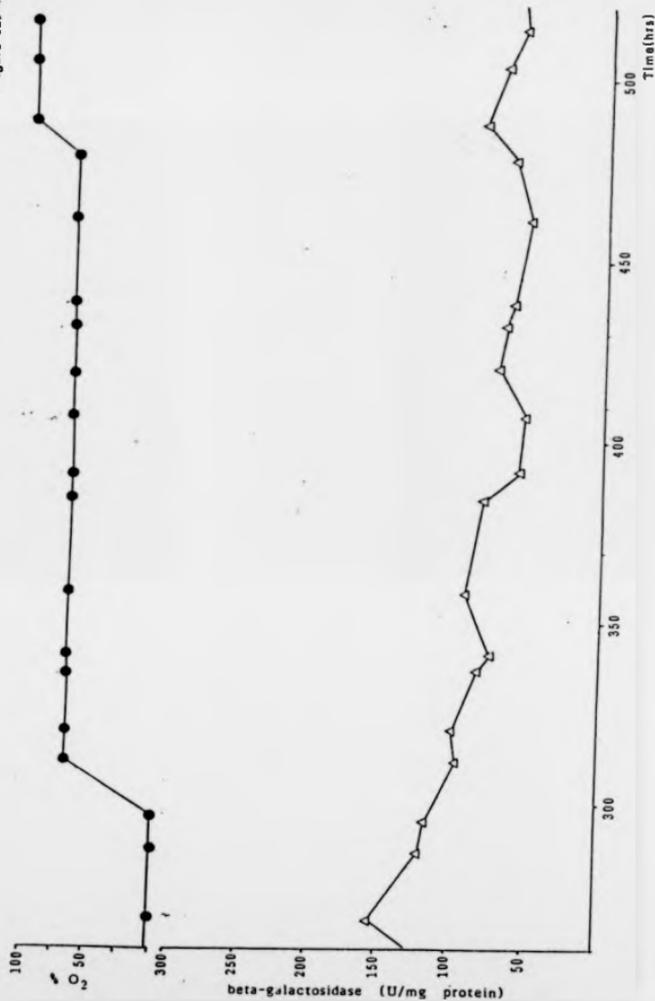
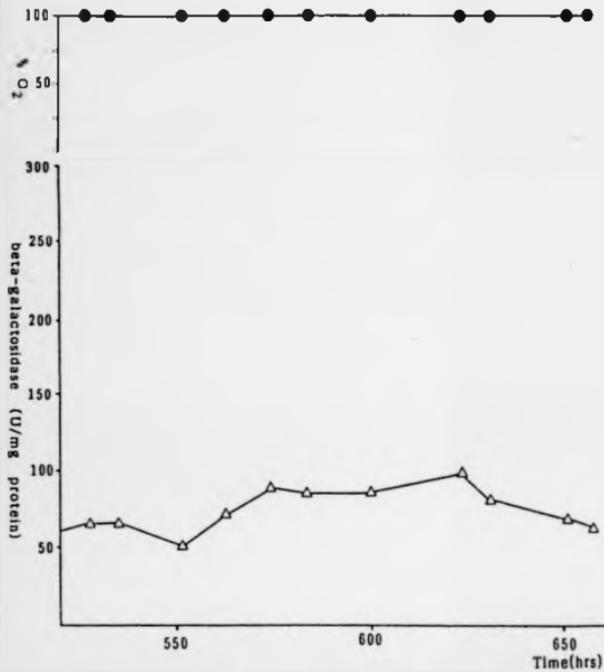


Figure 32.8



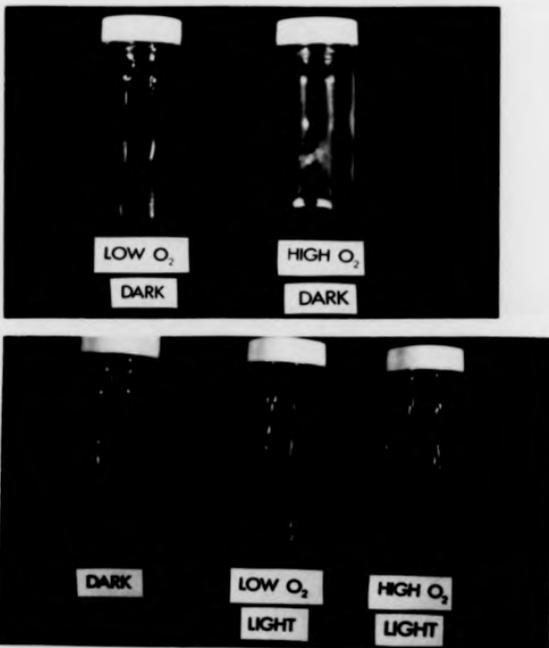
increased to 280 Units over 10 hours then fell to 95 Units over the next 25 hours, a steady state was achieved with the p^{QRS} activity varying between 95-150 Units over the next 115 hours. While maintaining light conditions the oxygen tension was increased to 60-70%. A steady state was achieved with p^{QRS} activity slowly dropping from 96 to 44 Units over the next 185 hours. The oxygen tension was increased to 100% and a steady state achieved with promoter activity slowly increasing from 60-100 Units over the next 140 hours. It was noted as oxygen tension increased the scatter of p^{QRS} activity values decreased.

5.7. Variation in the carotenoid content seen in continuous cultures.

During the previously described experiments in section 5.6. It was noted that the level of promoter activity did not always correlate with the apparent visible level of carotenoids accumulating in the cells of the culture. Figure 33 and Figure 34 show the colour of pelleted cells grown under different oxygen and light conditions. Figure 33 shows cells from experiment 5.6.2., cells grown in the dark under low oxygen tension are yellow in colour; and cells grown in the dark under enhanced oxygen tension are orange in colour. Figure 34 shows cells from experiment 5.6.4. cells grown under low oxygen tension in the dark are yellow in colour, cells grown under low oxygen tension in the light are orange in colour, and cells grown under enhanced oxygen tension in the light are dark red in colour. In order to qualify this observation the nature of the carotenoids being synthesised was investigated.

Figure 33. The colour of pelleted cells of *M. xanthus* exposed to high and low oxygen tensions in a dark grown continuous culture with a growth rate of 0.1 hr^{-1} :

Figure 34. The colour of pelleted cells of *M. xanthus* grown in a continuous culture with a growth rate of 0.1 hr^{-1} , under conditions of low oxygen tension in the dark; low oxygen tension in the light; and enhanced oxygen tension in the light:



5.7.1. Thin layer chromatographic examination of carotenoid extracts.

Carotenoids were extracted from the cells of cultures grown under dark conditions. These were developed on thin layer chromatograms as described in methods. A band of red pigment was present under high oxygenation which ran at an Rf of 0.91 this pigment was not present in the extractant of an identical wet weight of cells grown under low oxygenation. (results not shown).

5.7.2. Aluminium oxide column determinations of carotenoid extractions.

The efficiency of aluminium oxide column chromatography was examined using cells from a fully induced culture of M. xanthus. Carotenoid extractions were attempted from both wet and lyophilized cells. Due to problems resuspending the pellets in solvent, lyophilisation was abandoned. Two different solvent systems were used to extract carotenoids: 1) Acetone:Methanol (7:2); and 2) Methanol. The latter was used when the extractant was to be partitioned between phases with petroleum ether. Samples were developed on aluminium oxide columns (10cm x 1.5cm) activated with either 0.3% or 0.6% water. The column was developed with the following solvent sequence: 5ml petroleum ether (PE.); 10ml PE; 10ml PE; and 10ml PE plus 10% acetone. The fractions obtained were dried in a rotary evaporator, resuspended in hexane and scanned between 370 and 570nm. Scans did not reveal any triple peaks characteristic of carotenoids. Most of the material absorbed in the UV region below 300nm, the UV absorbing material could be removed by partition into PE. The large amount of red carotenoid seen in the extractant was not able to enter the

column.

Experimental samples were used in this system but no presentable data were obtained.

5.7.3. High performance liquid chromatographic determinations of carotenoid extractions.

Several extractions were developed using high performance liquid chromatography using conditions and columns described in the literature for the investigation of carotenoids (Methods 2.4.2.4.). Samples from experiment 5.6.2., and 5.6.4., and of identical wet weight were extracted using HPLC grade methanol. An accurately measured volume of this sample was scanned between 370nm-570nm. The volume of the remaining extractant was reduced under a stream of nitrogen and loaded onto the HPLC column. Figure 35 shows a histogram indicating the area under the curve calculated from the scan in methanol between 370-570nm. An increase in methanol soluble pigment is seen progressing from: 1) Dark and low oxygenation; 2) Light and low oxygenation; to 3) Light and high or enhanced oxygenation. The latter two separate conditions gave roughly similar values. Representations of the data obtained from HPLC examination are shown in Figure 36. In the extractant from cells grown under low oxygenation and light there are a large number of peaks in both the visible and the UV region. The number of peaks seen decreases dramatically in extractants from cells grown under high oxygenation and a further decrease is seen in calls grown under enhanced oxygenation. A comparison of extractions of dark grown cells under high and low oxygen

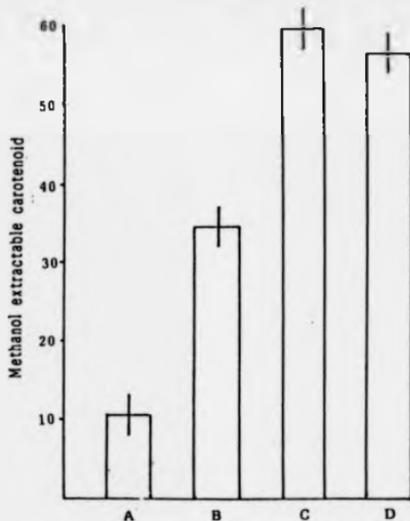
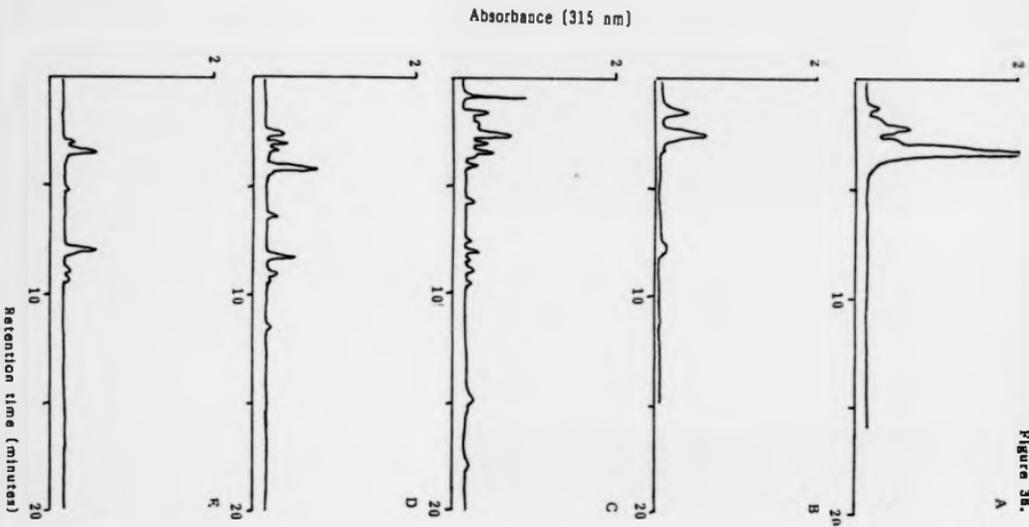


Figure 35. Histogram showing the amounts of methanol extractable carotenoid in cells of *M. xanthus* grown in continuous culture with a growth rate of 0.069hr^{-1} under different oxygen tensions, in the light and in the dark; The continuous culture was grown to a steady state in the dark at a low oxygen tension (A), then exposed to light (B), whilst maintaining the light stimulus the aeration was increased (C), the aeration was then further increased to give enhanced oxygenation (D).

Figure 30. HPLC analysis of carotenoids extracted from cells of M. xanthus grown in continuous culture with a growth rate of 0.1 hr^{-1} under different oxygen tensions, in the light and in the dark: Shows the HPLC analysis of identical wet weights of M. xanthus cells grown in continuous culture under different conditions of light and oxygen. A) Analysis of carotenoids from a low oxygenated culture in the dark; B) Analysis of carotenoids from a high oxygenated culture in the dark; C) Analysis of carotenoids from an illuminated low oxygenated culture; D) Analysis of carotenoids from an illuminated high oxygenated culture; E) Analysis of carotenoids from an illuminated enhanced oxygenated culture. Results for 315nm are shown, similar results were seen with the following wavelengths: 283, 340, 380, 380, 425, 450, and 470.

Figure 3b.
A



tension reveals that there is very little difference with only a small reduction in the number of peaks under high oxygenation.

5.8. Discussion of results

Under continuous culture steady state conditions in the absence of light, increasing oxygen tension results in an increase in expression from the p^{QRS} promoter. This was seen in four separate experiments. The aeration of a M. xanthus culture, with a growth rate of 0.05 hr^{-1} under dark conditions was increased approximately three-fold; this resulted in an approximately two-fold increase in p^{QRS} activity. The aeration of a M. xanthus culture, with a growth rate of 0.1 hr^{-1} under dark conditions was increased approximately four-fold; this resulted in an approximately two-fold increase in p^{QRS} activity. The increase in promoter activity was shown to be significant using Student's T-test. Additional evidence confirming the effect of oxygen on promoter expression came from a third experiment (5.6.1.). In this experiment an increase in p^{QRS} activity was seen after a three-fold increase in aeration. However, steady state conditions were not achieved in this experiment. In a fourth experiment p^{QRS} activity was measured in the dark under steady state conditions of limiting (1-2%), high (80-85%), and enhanced (100%) oxygen tension. This resulted in p^{QRS} activities of 1-3, 4.5-8.5, and 7-8.5 respectively. In the above experiments the need for a light stimulus to increase p^{QRS} activity has been removed. This illustrates that an oxygen species of some form is likely to be involved in the primary photoreceptive event.

The effects of light and oxygen together were studied. It was shown that the increase in p^{QRS} activity stimulated by light was dependent on

the level of oxygenation of the culture. The largest increases in p^{ORS} activity from identical light stimuli are seen in the cells of continuous cultures grown under conditions of high oxygenation. Under limiting oxygen tensions the level of promoter activity induced by the light stimulus gradually decreased to a value approximately 3 units above the basal dark level. This confirms that the photoinduction is dependent on the generation of an oxygen species. It is likely that under limiting oxygen tension this oxygen species may be quenched by very low levels of carotenoid.

Oxygen has an effect in addition to determining the level of promoter induction, it also determines the type of carotenoid that accumulates in light induced cells. A culture of M. xanthus grown under low oxygen tension was exposed to light, while maintaining the light stimulus the oxygen tension was increased. This was found to have very little effect on the measurable expression of the p^{ORS} promoter, however high oxygenation promoted the accumulation of a dark red pigment(s). This pigment(s) was observed on TLC, however, time did not permit an extensive determination of its identity. The HPLC conditions used did not develop the red pigment, however, a decrease in the complexity of eluent was noted as oxygenation increased. This is possibly due to a stoichiometric shift in the pigment profile towards that of the red pigment and away from the less hydrophilic molecules observed under low oxygen tension. If the red pigment could be isolated it would be expected that the decrease in the complexity of carotenoids within the cell would be accompanied by a concurrent increase in the amount of red pigment. Indeed it was noted that on increasing aeration the amount of methanol soluble pigment increased when comparing low oxygenation with high oxygenation, representing the enhancing effect of oxygen on promoter

activity described above, however, enhanced oxygenation appeared to yield approximately similar amounts of methanol extractable pigment to high oxygen.

The structure of the predominant carotenoids in M. xanthus, myxobacton and myxobactin, has been determined (Kleing et al., 1970; Reichenbach and Kleing, 1971). Myxobacton which represents up to 70% of the carotenoid content contains a keto group attached to the ring; both myxobacton and myxobactin are linked to a fatty acid glucose moiety through an oxygen atom. It is possible that these structures may represent the points of control at which oxygen affects carotenoid accumulation. An analogous system has been reported in Rh capsulatus in which oxygen causes the activation of a specific oxygenase which converts the predominant carotenoid under anoxic conditions spheroidene, to spheroidenone which contains a keto group in its ring (Shneour, 1982).

It should be noted that the lack of increase in p^{ORS} activity in a light induced culture when shifted to enhanced oxygen tension does not contradict the proposal that oxygen is a stimulator of promoter activity. It has been shown that light will not induce expression in a Car^C mutant (D. Hodgson pers comm). It is proposed that the response in this mutant is saturated making it unable to respond to the light stimulus. In a light induced culture it is likely that the response may become saturated making it unable to respond to the additional oxygen stimulus. Additional evidence to support this hypothesis comes from the observation of an accidental increase in the oxygenation of a light induced culture under low oxygen tension. The increase in oxygenation resulted in a 20-fold increase in p^{ORS} activity. In this case the low oxygen tension prevented saturation of the response by light allowing it to respond to the increase in oxygenation.

One other feature of note determined under continuous culture, is the observation of the scatter of p^{QRS} activities seen under high and low oxygen tensions, in both the light and the dark. It is interesting to note that there is a pronounced increase in the variation in p^{QRS} activity when grown under low oxygen tensions. This may be explained in that under low oxygen tension it is likely that the lethal event, the generation of toxic oxygen species, is less stringent than under high oxygen tensions. Thus allowing a greater variation in the protective response, namely the synthesis of carotenoids.

CHAPTER 6. Further studies of the mechanism of p^{QRS} induction in Myxococcus xanthus.

6.A.1. Further methods used in the study of carotenogenesis.

An effect of molecular oxygen on the p^{QRS} promoter has been established (Chapter 5). The nature of the interaction between oxygen and the promoter may be further investigated using a number of chemicals which are used to simulate, quench, and enhance possible oxygen metabolite signals. A number of these compounds are used to study photooxidation in cell free systems and may not be applicable to in vivo studies. The photooxidative product may be simulated by the direct addition of exogenous, reactive oxygen species such as hydrogen peroxide. Other reactive oxygen species may be generated by exogenous chemicals, either in the dark or upon illumination. For instance, methyl viologen (paraquat) generates the superoxide radical, in the dark. Photoactivation of acridine orange, acridine yellow, fluorescein, and lucifer yellow in cellular systems results in the production of singlet oxygen, hydrogen peroxide, and the hydroxyl radical (Martin and Logsdon, (1987). Rose bengal, methylene blue, and toluidine blue act via the generation of singlet oxygen (Davidson and Trethewey, 1977; Ito, 1978; Lang-Faulner and Rau, 1975).

There are several chemicals available that scavenge reactive oxygen species which may be used to quench a possible photooxidative product. Dimethyl sulphoxide, sodium benzoate, and thiourea act as hydroxyl scavengers. Singlet oxygen scavengers include histidine, 2-aminoethyl-isothiuronium bromide hydrobromide, 1,4-diazobicyclo [2.2.2] octane (DABCO), and beta-carotene (Davidson and Trethewey, 1977; Foote, 1988;

Quannes and Wilson, 1968; Peak et al., 1981). Mutants with defects in enzymes such as superoxide dismutase, or the addition of exogenous enzyme to a cell in which it is not present, may be used to implicate oxygen species. However proteins themselves may quench reactive oxygen species non-catalytically and so difficulty arises in interpreting data from the latter experiments.

The photooxidative product may be investigated by enhancing its effect. The singlet oxygen molecule is quenched by water molecules, this quenching is inhibited when the hydrogen atoms in the water molecule are replaced by tritiated hydrogen. However difficulties arise in replacing established hydration shells with tritiated water in living systems and so this approach cannot be used in this study.

8.A.2. Some examples of previous studies of carotenogenesis.

Hydrogen peroxide has been shown to replace the light signal and stimulate the synthesis of carotenoids in the dark in Fusarium aquaeductuum (Thalmer and Rau, 1970). The photoinduction was abolished in the presence of dithionate and hydroxylamine, leading to the conclusion that an oxidative step was involved in photoinduction. Lang-Faulner and Rau (1975) studied the effect of several redox dyes on the carotenogenesis of Fusarium aquaeductuum. They showed that only photoactive dyes could stimulate carotenoid synthesis in the presence of white light. The native receptor in Fusarium aquaeductuum absorbs in the blue region. It was shown that red light had no effect on the accumulation of carotenoids unless the dyes toluidine blue or methylene blue, which absorb maximally between 600-700nm., were present. These dyes stimulated carotenoid

accumulation to similar levels as white light. The carotenoids produced were qualitatively similar to those seen in white light, however quantitatively there were less of the more unsaturated carotenoids. A similar quantitative effect is also seen with the small levels of carotenoids synthesized under dark conditions and as such the difference is probably not due to photoinduction by the dyes. The photoreactive dyes may act either by providing the photooxidative product directly, or by passing captured photoactivation energy to the native receptor. Examples of the latter have been reported by Foote, 1970; and Mathis, 1972.

6.B.1. The link between light induced systems and the heat shock response.

There are a large number of genes defined as being induced by the heat shock response. The functions of the proteins they code for include DNA excision and repair, and chaperonins. Although defined as heat shock proteins a number of these may be induced by alternative stimuli (Mestril et al., 1986; Riddihough and Felham, 1986). In a study of heat shock proteins HSP68, HSP70, and HSP80, in Chlamydomonas reinhardtii, vanGromoff et al. (1989) showed that light alone caused the accumulation of mRNA's encoding these genes. The levels of mRNA's were equivalent or higher after heat shock, when compared with levels after exposure to light. The 22kD heat shock protein (HSP22) also in Chlamydomonas reinhardtii does not accumulate in the light unless prior heat shock has occurred (Jah-Shalom et al., 1990).

The interactions of three stress responses were studied in parsley cells (Walter, 1989): 1) the induction, by UV-light, of flavonoid glycosides, which probably have a photoprotective role; 2) the induction, by fungal cell

wall fragments, of furanocoumarins, which have a role in plant defence; and 3) the induction of heat shock proteins. It was discovered that the UV and fungal elicitor stress responses were overridden and decreased by the heat shock response, thus establishing a hierarchy of response. Goerlich et al. (1988) showed that hydrogen peroxide would induce the synthesis of heat shock genes in E. coli. This may illustrate a possible mechanism through which light could induce such genes; that being via the generation of toxic oxygen species as outlined in the Introduction 1.B..

8.1. Solid media spot tests of compounds which may affect carotenoid synthesis.

Plate spot tests were used as a rapid screen of chemicals which may affect carotenogenesis. Top lawns of M. zanthus in 0.75% agar were incubated with sterile 10mm diameter filter paper discs containing various compounds of interest. The chemicals used are listed in Table 1, along with their proposed effect on carotenogenesis. All chemicals were used at 500mg/ml or at saturation whichever was the lower. Hydrophobic chemicals will diffuse throughout the plate via cell wall adhesion in the absence of solvent, therefore the most volatile solvent that could be used was chosen. This reduces the possibility of solvent effects which were examined using solvent only discs.

Plates were incubated in replica under a variety of light and dark conditions. No chemicals were found that induced visible carotenoid accumulation in the dark. No chemicals were found to induce beta-galactosidase activity as measured by the chromogenic substrate X-Gal (see Methods 2.3.4.). In the study of possible inhibitors of light induced

carotenoid synthesis; if inhibition was occurring it could not be distinguished from zones of reduced growth at the edges of lysis rings, which appear pale in colour. The lysis rings being caused by inhibitory concentrations of the compound close to the filter disc.

Table 1. Chemicals, and there proposed activities, used in spot test experiment

Chemicals

Diphenylamine (DPA)	Prevents saturated carotenoid synthesis.
Nicotine	Prevents the formation of ring structures
Butylated hydroxytoluene (BHT)	Quenches radicals
1,4-diazobicyclo[2.2.2.] octane (DABCO)	Quenches singlet oxygen.
Hydroxylamine	Reducing agent.
Phenylacetic acid	Inhibits flavin synthesis.
Methyl viologen	Generates the superoxide radical.
Hydrogen peroxide	Substitutes for light generated H_2O_2 .

Solvents

Sterile distilled water
Ether
Toluene

6.2. The effect of exogenous reactive oxygen species, and generators of reactive oxygen species in the dark.

6.2.1. The effect of exogenous hydrogen peroxide on p^{QRS} activity.

To confirm the results seen with solid media, and to enable small alterations in promoter activity to be detected, the effect of addition of hydrogen peroxide to batch cultures was studied. Such small promoter activities would probably not be seen in the crude X-gal assay, or be represented as a visible accumulation of carotenoids, they may however be of biological significance.

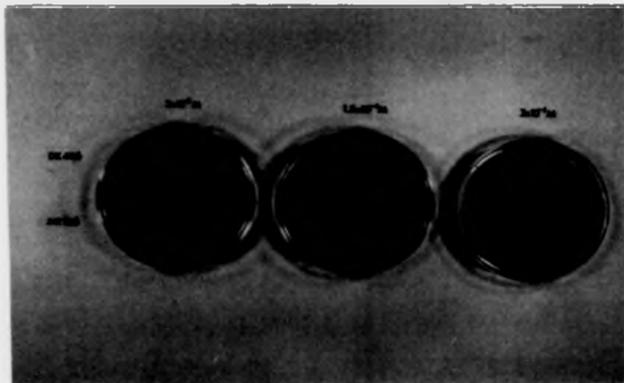
Three 250ml cultures were maintained in the dark. When an optical density of around 0.1-0.2 Units was achieved, H₂O₂ at concentrations of 0.001 M, and 0.0001 M, was added, a control to which no H₂O₂ had been added was maintained under identical conditions. H₂O₂ at a concentration of 0.001 M partially inhibits growth of M. xanthus, whereas at a concentration of 0.0001 M no effect on growth rate is seen. Promoter activity was assayed as per Methods 2.3.2. throughout the cultures growth cycle. Results (not shown) indicated only a small variation in promoter activity, with no significant increase being seen after the addition of hydrogen peroxide. Cells grown in the presence of H₂O₂ (0.001 M) expressed the p^{QRS} promoter at activities between 8-4 Units, and at 0.0001 M activities between 4-11.5 Units. These values are not significantly different from the culture to which no hydrogen peroxide was added which showed a variation in p^{QRS} activity between 8-12 Units

8.2.2. The effect of methyl viologen on p^{QRS} activity.

Preliminary experiments were carried out to determine a suitable concentration of methyl viologen at which to examine p^{QRS} activity. Top lawn spot tests showed methyl viologen has an inhibitory effect at concentrations of around 1×10^{-4} M and above. Cells treated with 3×10^{-5} M and below showed no effect. Previous experiments had shown discrepancy between the effects of chemicals in liquid, and on solid media. To allow for any differences between the effect of a compound in liquid and in solid media, a kill curve was also performed using liquid cultures. A concentration of 1.3×10^{-4} M was chosen, which inhibits growth slightly. Cells grown in the presence of 1.3×10^{-4} M methyl viologen exhibit a growth rate of 0.08 hr^{-1} , as opposed to cells of the control culture lacking methyl viologen which exhibit a growth rate of 0.16 hr^{-1} . Additional preliminary experiments were carried out to determine if the presence of carotenoids in a constitutive producing strain (Car^{C}) would confer an advantage in the presence of methyl viologen, over a strain unable to synthesise carotenoids (Car^{-}). When grown on solid media in the presence of increasing concentrations of methyl viologen it was discovered that the Car^{C} strain was able to grow on 3×10^{-4} M of methyl viologen whereas growth of the Car^{-} strain was inhibited (Figure 37).

p^{QRS} activity was measured in cells grown in three 250ml cultures, in the dark. Aeration was provided by means of an orbital shaker at 30°C . One culture contained 1.3×10^{-4} M of methyl viologen at the time of inoculation. A second was grown to an OD_{660} of 0.13, at which point methyl viologen was added to a concentration of 2.8×10^{-4} M. The third culture was a dark grown control. Methyl viologen showed very little

Figure 37. Growth of Car⁻ (MR135) and Car^C (DK406) strains on solid media containing a range of paraquat concentrations:



effect on promoter activity while having a marked effect on growth rate and culture morphology. p^{QRS} activity in the presence of 1.3×10^{-4} M methyl viologen varied between 4-14 Units throughout the growth cycle of the culture. p^{QRS} activity in the presence of 2.6×10^{-4} M methyl viologen varied between 8-13 Units. These values were similar to those measured in cells of the control culture, in which p^{QRS} activity varied between 8-18 Units throughout the growth cycle. A dark brown pigment was secreted into the medium in cultures containing methyl viologen. The pigmentation was visibly darker in culture containing the highest concentration of methyl viologen. Microscopic examination revealed changes in cell morphology. The rods became more rounded in the centre and there were approximately twice the number of filamentous cells, at twice the normal cell length, per field as compared with the control culture. In addition there was a four fold increase in the number of spheroplasts seen in the presence of methyl viologen.

6.3. The effect of reactive oxygen species quenchers on p^{QRS} expression.

The quenchers considered for use in this study were hydroxylamine, butylated hydroxy toluene, and DABCO. Of these, butylated hydroxytoluene was too insoluble in aqueous solution to be of any practical use. There was insufficient time to study the effects of both DABCO and hydroxylamine, so only the effects of DABCO were studied. The range of concentrations of DABCO at which to examine p^{QRS} activity was determined in preliminary experiments on solid media, then more accurately in liquid media. In liquid media DABCO at 0.008 M has no effect on growth rate, at 0.0125 M a slight inhibition was seen.

6.3.1. The effect of DABCO on p^{QRS} expression.

The effect of DABCO on the level of induction of p^{QRS} activity by identical white light treatments was determined. A 250 ml culture of M. xanthus containing 0.01 M was grown to an OD_{660} of 0.35 then exposed to light at $120 \text{ microEm}^{-2}\text{s}^{-1}$. In the presence of DABCO, the rise in p^{QRS} activity seen after exposure to light was delayed by two hours. In addition the rate of increase in p^{QRS} activity was slower in the presence of DABCO, being 0.55 Units/minute in the presence of DABCO, and 0.83 Units/minute in the control culture. Both cultures exhibited identical growth rates therefore excluding the possibility that inhibition of p^{QRS} activity is being caused by a general inhibition of the metabolism of the cell (see Figure 38).

Cultures of M. xanthus were exposed to a range of concentrations of DABCO (0.009 M, 0.01M, 0.012M) after exposure to light. Figure 39 shows addition of DABCO one hour after light induction, Figure 40 shows addition of DABCO three hours after light induction. Addition one hour after light induction shows the degree of inhibition of promoter induction increasing as the concentration of DABCO increases. Addition of DABCO three hours after light induction showed little effect on promoter activity except for an enhanced activity seen in the presence of 0.009 M. The enhanced response was also seen in a culture to which 0.009 M DABCO was added one hour after light induction. The presence of DABCO showed a degree of inhibition of p^{QRS} induction in the early stages of the growth cycle of the culture, however, in the later stages induction of p^{QRS} appeared to be enhanced. This effect was only seen with the lowest concentration of DABCO.

Figure 38. The effect of growth of M. xanthus in the presence of 0.01 M DABCO on the level of induction of p^{QRS} by light: (A) \diamond) p^{QRS} activity in the absence of DABCO in a batch culture exposed to light; \triangle) p^{QRS} activity in the presence of 0.01 M DABCO in a batch culture exposed to light; \blacklozenge) p^{QRS} activity in the absence of DABCO in a batch culture maintained under dark conditions; \blacktriangle) p^{QRS} activity in the presence of DABCO in a batch culture maintained under dark conditions. (B): the optical density corresponding to the cultures exposed to light plotted on a logarithmic scale. \diamond) in Λ_{880} of light grown M. xanthus in the absence of DABCO; \triangle) in Λ_{880} of light grown M. xanthus in the presence of DABCO.

Figure 39. The effect of the addition of a range of concentrations of DABCO one hour after the light stimulus, on light induction of p^{QRS} . The point at which the cultures were exposed to light is marked by an arrow, which also indicates the light intensity in $\mu\text{E m}^{-2} \text{ s}^{-1}$. \blacklozenge) 0.005 M DABCO; \blacktriangle) 0.01 M DABCO; \square) 0.0125 M DABCO; \triangle) control, no DABCO.

Figure 40. The effect of the addition of a range of concentrations of DABCO three hours after the light stimulus, on light induction of p^{QRS} . The point at which the cultures were exposed to light is marked by an arrow, which also indicates the light intensity in $\mu\text{E m}^{-2} \text{ s}^{-1}$. \blacklozenge) 0.009 M DABCO; \blacktriangle) 0.01 M DABCO; \square) 0.0125 M DABCO; \triangle) control, no DABCO.

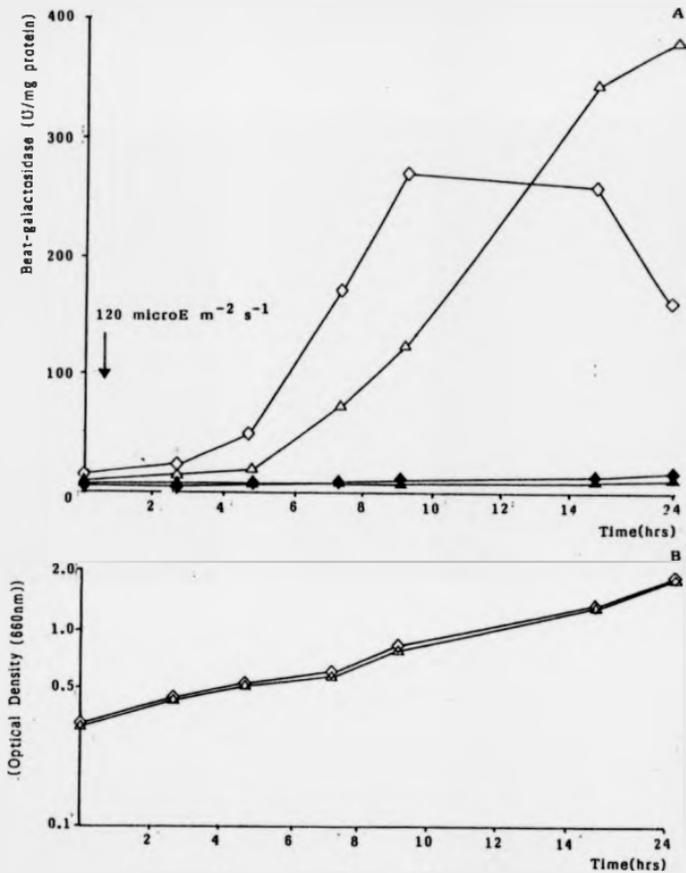


Figure 30.

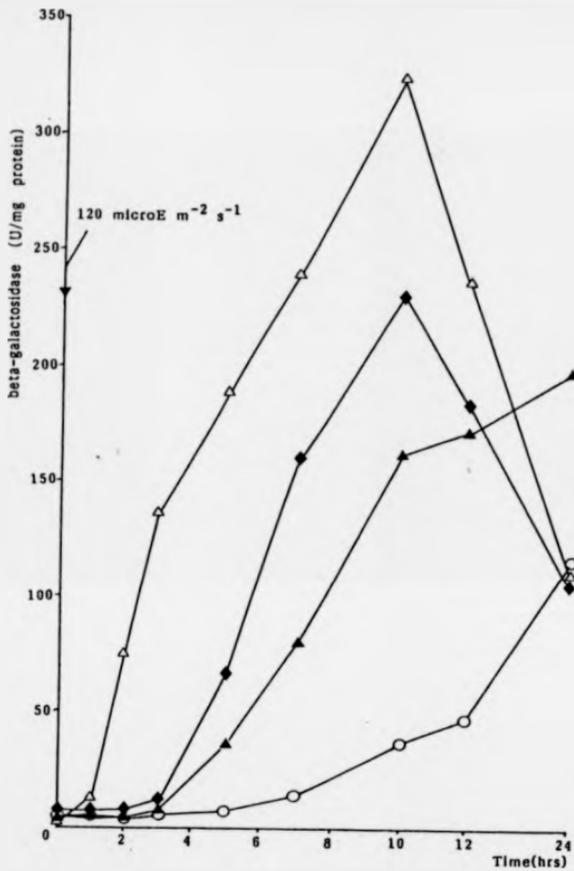


Figure 39.

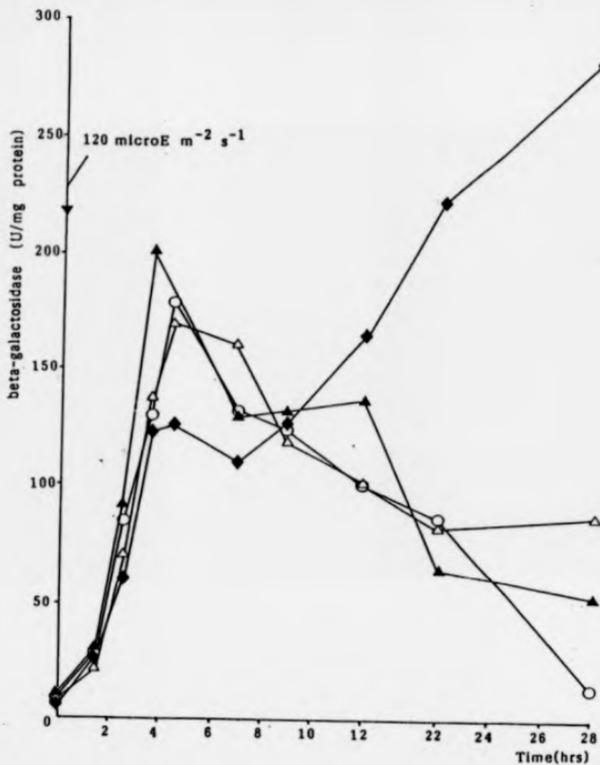


Figure 40.

8.4. The effect of photoactivated dyes on p^{QRS} expression.

8.4.1. Induction of p^{QRS} expression by toluidine blue.

The main criteria used to choose a suitable dye for use in this study was that the dye must not have an absorption maxima in the blue region. This allows the effects of the exogenous dye to be distinguished from those of the native photoreceptor, protoporphyrin IX. Toluidine Blue had previously been used in a similar experiment (Lang-Faulner and Rau, 1975) and was chosen for this study, as it generates singlet oxygen on illumination with red light. Preliminary experiments showed that toluidine blue at a concentration of 200pM inhibited growth, 100pM showed no effect on the cultures growth rate and was used in these experiments.

Figure 41 shows the p^{QRS} activity in the presence of toluidine blue under different light regimes and in the absence of the dye as controls. White light was at an intensity of $120 \text{ microEm}^{-2}\text{s}^{-1}$, red light provided by a red filter was at an intensity of $35 \text{ microEm}^{-2}\text{s}^{-1}$ (see Appendix B for transmission spectrum of red filter). *M. xanthus* was grown in 250 ml cultures either un-supplemented, or containing 100pM toluidine blue.

When exposed to white light cells grown in cultures containing 100pM toluidine blue showed an increase in p^{QRS} activity throughout the cultures growth curve to a value 3.5-fold higher than p^{QRS} activity measured in cells of the control culture, exposed to white light in the absence of toluidine blue. In the presence of toluidine blue promoter activity was not inhibited towards the end of the growth curve, as is seen routinely in white light induced cultures. p^{QRS} activity in the dark is the same in the presence of 100pM toluidine blue, and the absence of the dye. The culture containing toluidine blue which was exposed to red light showed a greater

Figure 41. The effect of toluidine blue on the induction of p^{QRS} by white and red light: ■) p^{QRS} induction by white light; ◇) p^{QRS} induction by red light; ○) p^{QRS} induction by white light in the presence of 100pM toluidine blue; ▲) p^{QRS} induction by red light in the presence of 100pM toluidine blue. The p^{QRS} activity seen in the presence of red light is equivalent to that measured in dark grown cells in both the presence and absence of toluidine blue.

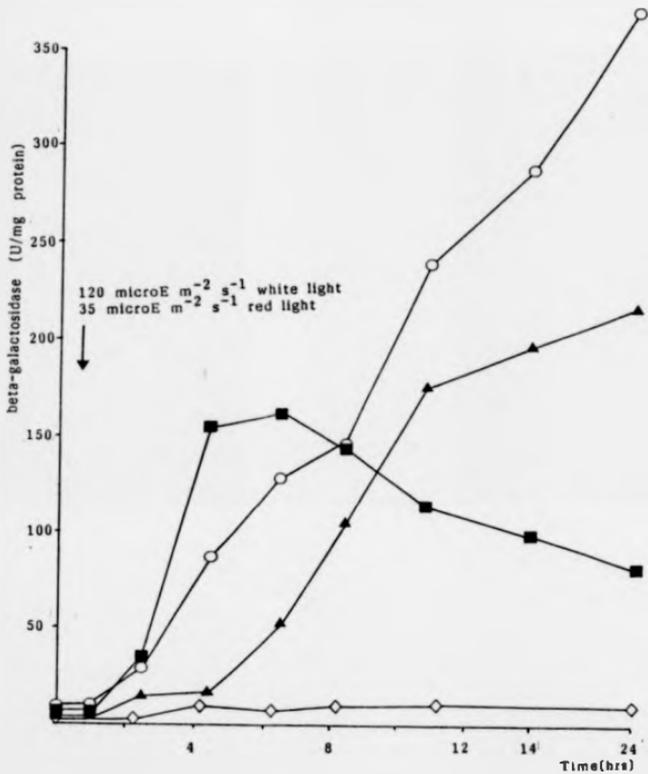


Figure 41.

lag in the onset of p^{QRS} expression of 4 rather than 2 hours seen in cells of a culture exposed to white light in the absence of the dye. After the lag period p^{QRS} activity, in cells exposed to red light in the presence of toluidine blue, rose to a level similar to the unsupplemented culture exposed to white light. Again unlike the unsupplemented culture there is no decrease in promoter activity towards the end of the growth curve. Red light in the absence of toluidine blue had no effect in inducing p^{QRS} activity.

8.4.2. The effect of singlet oxygen quencher, DABCO, on p^{QRS} induction by toluidine blue.

The possibility exists that toluidine blue is acting directly on the protein responsible for the translation of the light signal to the p^{QRS} promoter, the proposed function of the CarR protein. To confirm that a singlet oxygen intermediate is required for the induction of p^{QRS} by toluidine blue, similar experiments were carried out to those described in section 6.3.1., whereby the singlet oxygen signal is quenched using DABCO. The effect of a range of DABCO concentrations on the induction of p^{QRS} by white light in the presence of toluidine blue was studied (Figure 42). DABCO was added to concentrations of 0.009 M, 0.01 M and 0.0125 M, one hour after the light stimulus was applied to the M. xanthus cultures. In the absence of DABCO p^{QRS} activity increased to a peak of around 245 Units, after which it is decreased showing that unlike in previously described experiments (Section 8.4.1.) the signal from toluidine blue undergoes what is presumed to be quenching by the accumulating carotenoids. In the presence of DABCO, added one hour post light induction, degrees of

Figure 42. The effect of the addition of a range of concentrations of DABCO one hour after the light stimulus, on the white light induction of p^{QRS} in the presence of toluidine blue: The point at which the cultures containing 100pM toluidine blue were exposed to white light is marked by an arrow, which also indicates the light intensity in $\text{microE m}^{-2} \text{s}^{-1}$. Δ) 0.009 M DABCO; \blacktriangle) 0.01 M DABCO; \diamond) 0.0125 M DABCO; \circ) control, no DABCO.

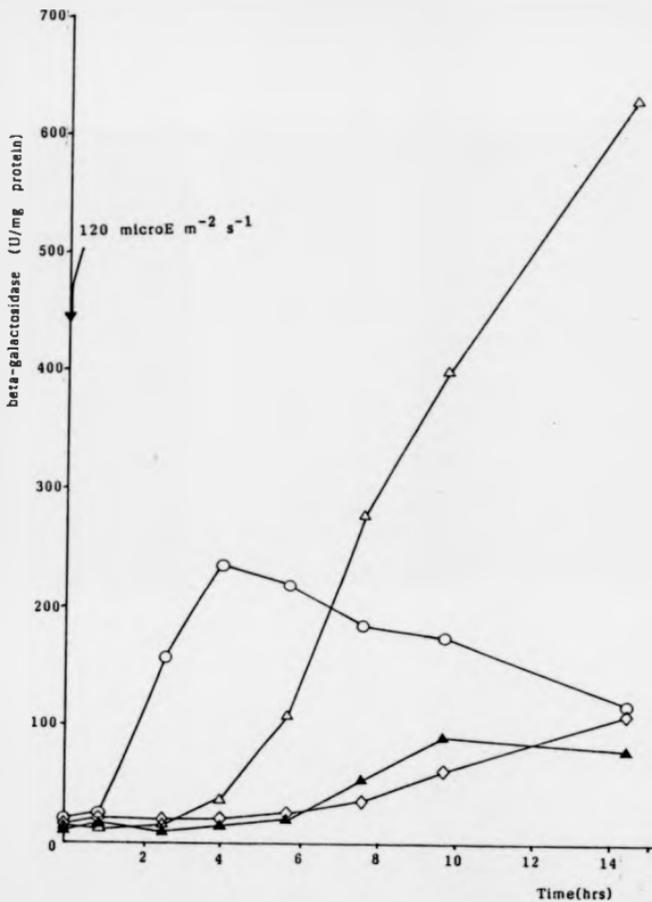


Figure 42.

quenching of the induction of p^{QRS} by light were seen. Again at the lowest concentration of DABCO (0.009 M), p^{QRS} induction is quenched during the early stages of the cultures growth cycle and enhanced during the latter stages. It should be noted that the combination of toluidine blue, white light, and DABCO at concentrations of 0.01 M and 0.0125 M caused an inhibition in growth rate.

The ability to quench the induction of p^{QRS} shows that both the native photoreceptor, protoporphyrin IX, and the exogenous photosensitizer, toluidine blue, are acting via singlet oxygen. A final confirmation that toluidine blue was acting via singlet oxygen in inducing p^{QRS} was shown by studying the effect of 0.01 M DABCO added one hour after induction of p^{QRS} by red light in the presence of 100pM toluidine blue (Figure 43). The addition of DABCO causes a marked inhibition of the level to which red light induces p^{QRS} activity in the presence of 100pM toluidine blue. In this instance both cultures showed identical growth rates.

6.5. The effect of heat shock on p^{QRS} expression.

Sequence analysis of p^{QRS} showed it had 50% homology to the E. coli consensus heat shock promoter. This raised the possibility that the induction of carotenoids in M. xanthus was part of a network of stress induced responses which shared some common regulatory elements. To determine if this were so the effect of heat shock on expression from the p^{QRS} promoter was determined.

Promoter expression was examined in 250ml cultures grown under dark conditions. Heat shock was provided by the transfer of the culture to a shaking water bath at 40°C, the control culture was transferred to a water

Figure 43. The effect of the addition of DABCO one hour after the light stimulus, on red light induction of p^{QRS} . The point at which the culture containing 100pM toluidine blue were exposed to red light is marked by an arrow, which also indicates the light intensity in $\text{microE m}^{-2} \text{s}^{-1}$. \diamond) 0.01 M DABCO; \bullet) control, no DABCO.

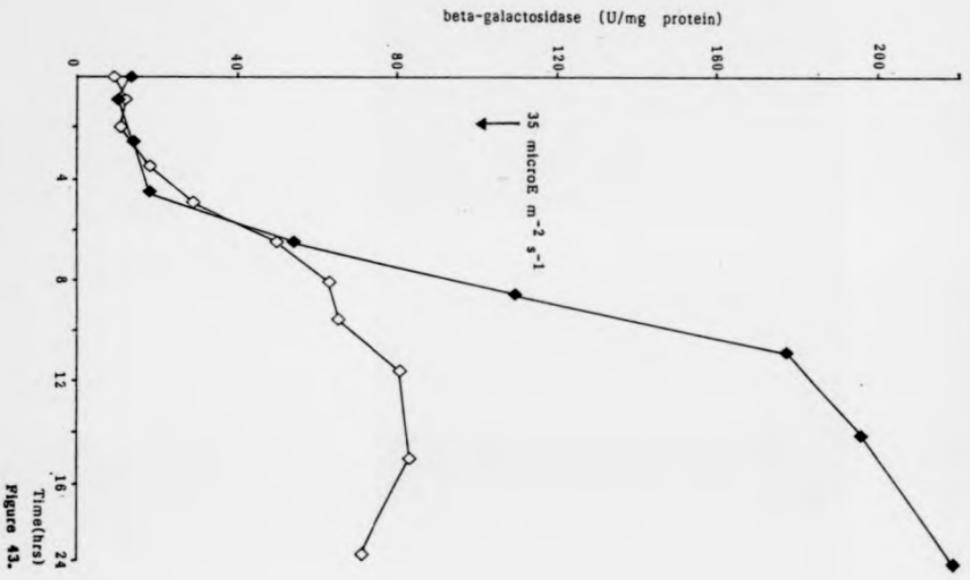


Figure 43.

bath at 30°C. Colony forming units were determined before and after the transfer. The effect of heat shock was determined in two experiments, one using a culture that had an identical optical density as the control, and a second experiment in which the heat shock culture with an optical density twice that of the control was used. The former was to remove any possible growth phase effects that might be seen. The latter was to allow for the extensive cell death reported after heat shock in order that both cultures would have a similar number of cells post-heat shock.

In both experiments heat shock resulted in a 50% decrease in colony forming units. This is in agreement with published data (Killeen and Nelson, 1988). In both experiments no increase in promoter activity was seen after heat shock, however p_{QRS} activity of the heat shock culture remained 3-10 units above that of the control culture (results not shown).

6.6. Discussion of results.

To study the effect of various chemicals on carotenogenesis in M. xanthus spot tests of the chemical on bacterial top lawns were studied under light and dark conditions. This approach proved unsatisfactory for the following reasons: 1) deficiency in carotenogenesis could not be distinguished from inhibition of growth by these chemical; 2) X-gal and MUG proved insufficiently sensitive to detect any possible variation in p_{QRS} activity; 3) M. xanthus grows very poorly in top agar requiring a high cell density inoculum which may mask any slight phenotypic alteration in pigmentation seen. To overcome this problem more lengthy, and possibly more variable, batch cultures were employed.

The addition of methyl viologen, a superoxide generator, to a growing

culture of M. xanthus maintained in dark conditions, or the growth of M. xanthus in the presence of methyl viologen in the dark had no effect on the activity of the p^{QRS} promoter. Addition of hydrogen peroxide, at both inhibitory and sub-inhibitory concentrations, to a dark grown culture did not alter the activity of the p^{QRS} promoter. These two results suggest that neither hydrogen peroxide, or the superoxide radical, are involved in the primary photochemical event in the induction of the p^{QRS} promoter. The two major oxygen species that remain are the hydroxyl radical and the singlet oxygen molecule. As has been outlined in the Introduction the consensus of opinion is that porphyrin, and more specifically protoporphyrin IX, photosensitization in the presence of molecular oxygen proceeds primarily via a singlet oxygen mechanism. A generator of hydroxyl radicals was not used in this study. It is believed that the high and non specific reactivity of the hydroxyl radical make it an unlikely specific inducer. However, had time permitted the effect of a quencher of the hydroxyl radical, butylated hydroxytoluene, on p^{QRS} induction would have been studied.

The role of singlet oxygen in the induction of the p^{QRS} promoter was studied in two ways. Firstly, the singlet molecule was removed from the induction process by a highly efficient quencher, DABCO. This would mimic the proposed action of carotenoids. Secondly, the effect of a photoreactive dye, toluidine blue, which specifically produces singlet oxygen, on p^{QRS} activity was studied. It was shown that the level of induction of p^{QRS} was reduced under identical light conditions by the addition of the quencher DABCO. The degree of reduction in p^{QRS} activity was proportional to the concentration of DABCO. The quenching was more efficient when the quencher was added one hour post light stimulus, as

compared to three hours. This is to be expected as carotenoids are seen to accumulate at around 2-3 hours post light stimulus, indicating the events responsible for the accumulation of carotenoids should occur before three hours post light stimulus.

The treatment of a culture of *M. xanthus* with white light in the presence of toluidine blue produced an induction in p^{QRS} activity which was three-fold higher than the induction of p^{QRS} by white light in the absence of toluidine blue, i.e. due to endogenous photosensitizer only. Despite the presence of submaxima in the absorption spectrum of Protoporphyrin IX in the red region, it was shown that filtered red light at $35 \text{ microE m}^{-2} \text{ s}^{-1}$ did not induce p^{QRS} activity. However, in the presence of toluidine blue red light induced p^{QRS} activity to a level roughly similar to that seen in cells exposed to white light at $120 \text{ microE m}^{-2} \text{ s}^{-1}$ in the absence of the exogenous photosensitizer. This illustrates that the induction of p^{QRS} activity by red light is specific to toluidine blue, and that the dye is not simply enhancing induction by the endogenous photosensitizer. It should be stressed that toluidine blue may act via the generation of singlet oxygen, or through a type I reaction. To confirm that toluidine blue is inducing the p^{QRS} promoter via a singlet oxygen intermediate the effect of the singlet oxygen scavenger DABCO on the level of induction in the presence of toluidine blue was studied. Induction of p^{QRS} by white light and red light, in the presence of toluidine blue, was quenched by DABCO, thus confirming that toluidine blue is acting through a singlet oxygen intermediate in inducing the p^{QRS} promoter.

Two lines of evidence suggested that the p^{QRS} promoter may form part of a general stress response, which would include environmental stresses such as heat shock. Firstly, the sequence of the promoter region

which showed that the p^{QRS} promoter was 50% homologous with to the E. coli stress related consensus promoter. Secondly, a number of light inducible genes have also been shown to be induced by the heat shock response (as discussed in the Introduction 6.B.1.). Heat shock conditions for M. xanthus have previously been described and were applied in this study. However, it was shown that heat shock did not induce p^{QRS} activity. This is in agreement with the action of hydrogen peroxide which has been shown to be an excellent inducer of the stress response.

CHAPTER 7. Conclusions.

7.1. General conclusions and discussion.

The aims of this project were as follows: to establish a heterologous system in which light may be used to control the expression from the p^{QRS} promoter; to use this system to determine the requirements for light inducible gene expression, and to establish its possible use in other heterologous systems eg. Streptomyces species; to establish the nature of the primary photo-inductive event in the induction of the p^{QRS} promoter in M. xanthus; and to characterize the physiology of the light inductive process in M. xanthus.

Expression from three different sized fragments of the p^{QRS} promoter region was examined in E. coli. Two very different profiles were obtained: E. coli strains containing the 5.8kb (Hind III/Bgl II), and the 1.2kb (Sma I/Bgl II) fragments of the p^{QRS} region expressed beta-galactosidase activities of around 700 Units. In the presence of the 5.8kb Stu I fragment, which contains the entire carQRS region, expression from these regions of DNA was decreased. This decrease in expression was specific to the fragment spanning the carQRS region, as introduction of 5.8kb of unrelated DNA did not reproduce the repression seen. Plasmids containing the 0.4kb (Sac I/Bgl II) fragment of the p^{QRS} region expressed beta-galactosidase activities at a lower level of around 300 Units. This lower activity was not due to promoter mutation as the 0.4kb fragment produced wild-type carotenoid expression in M. xanthus. Introduction of the 5.8kb Stu I fragment containing the carQRS region, resulted in an orientation specific activation of expression from the promoter region as measured by

beta-galactosidase activity.

The question as to which of these expression profiles, if any, represents that seen in M. xanthus, i.e. shows expression of the p^{QRS} promoter alone, can be partially answered at present. Several lines of evidence suggest that, contrary to initial beliefs, it is expression from the 0.4 kb p^{QRS} fragment which represents true expression of the p^{QRS} promoter. The first of these is the confirmation that full promoter activity may be obtained from regions within the 0.4kb p^{QRS} fragment in M. xanthus (S.McGowan pers. comm.). The second comes from reconstitution experiments, whereby regions of the carQRS region were introduced into strain MR135, which contains a deletion spanning the entire region. These studies showed a factor previously unidentified was required for expression of p^{QRS}. When the p^{QRS} promoter alone is present, i.e. in the absence of the carR gene product, the proposed repressor, expression was found to be at a basal level of around 5 units. This basal level was increased by around four-fold in the presence of light by the introduction of the entire carQRS region. Having previously established that neither gene products from carB or carS could act as an activator of the p^{QRS} promoter (D.Hodgson pers. comm.) this suggested that some other factor expressed by the carQRS region was stimulating expression in the presence of light. Further analysis in D.Hodgson's laboratory has shown that the product of carQ gene is required for full promoter activity (McGowan and Gorham, pers. comm.). From this result it would be expected that in the absence of the carQ gene product, expression from p^{QRS} in E. coli would be at a basal level. This is seen only with the 0.4kb promoter fragment. It is possible that the increase in expression, from the 0.4kb fragment, seen after the introduction of the entire carQRS region, in one orientation, may

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be due to the presence of the carQ gene product, thus providing a further indication that the 0.4kb fragment is expressing the p^{QRS} promoter. The lack of activation when the carQRS region is orientated in the opposite manner may be due to an orientation specific inhibition of expression from the carQ gene.

Sequence analysis of the carR gene showed it to have the marked characteristics of a membrane bound protein, and more importantly to lack any of the previously identified DNA binding motifs. This made the promoter exclusion repression previously envisaged in the control of p^{QRS} in M. xanthus unlikely. This was further evidence that the repression seen in expression from the larger fragments of the p^{QRS} region was not due to the same mechanism that occurs in M. xanthus. The questions remain as to what it is that causes high levels of expression from the larger p^{QRS} fragments, and causes the repression of that expression in the presence of the carQRS region.

It is possible that the E. coli RNA polymerase is recognizing an alternative promoter-like sequence, termed p^X, in the region of DNA upstream of the Sac I site, and is only poorly recognizing the actual p^{QRS} promoter sequence. This poor recognition may be caused by the lack of the carQ gene product. The mechanism of repression seen with these constructs remains to be established. It may be that the additional copy of the p^X promoter region, when introduced as part of the entire carQRS region sequesters a limiting factor required for expression of the promoter. Thus overall expression from p^X upstream of the lacZ gene is reduced.

The switch between dark and light growth conditions had no effect on the expression profiles of any of the promoter probe vectors used in this study. This may be due to the lack of some element required for the

transduction of the light signal to the promoter in the heterologous system. As postulated above it is likely that the 0.4kb p^{QRS} fragment shows expression from the p^{QRS} promoter, which may be activated in the presence of the carQRS region, possibly via the carQ gene product. As such the potential of E. coli as a tool with which to study the mechanism of induction of p^{QRS} , where use of M. xanthus is not applicable or prohibitive, still remains.

The effect of increased uptake of the proposed photosensitizer, protoporphyrin IX, on expression of the promoter in E. coli was examined. This was the first step in attempting to reestablish light induced gene expression in E. coli by the addition of factors required for the transduction of the light signal to the p^{QRS} promoter. Wild type E. coli is impermeable to protoporphyrin IX. To overcome this an NTG mutagenised strain was selected in which it could be shown that protoporphyrin IX uptake was occurring. This strain was transduced with the promoter probe plasmids. It is suggested that the plasmids used in this study do not predominantly express the p^{QRS} promoter, but do, however, express the p^X promoter. As such expression profiles had they been obtained would have been unlikely to have a direct relevance to the mechanism by which p^{QRS} is induced by light. The protoporphyrin IX permeable strain was found to be light sensitive in the presence of protoporphyrin IX. Light decreased promoter activity from the promoter probe plasmids, this decrease was accompanied by cell death. Photolysis in the presence of protoporphyrin IX occurs in the absence of carotenoid synthesis in M. xanthus. Therefore, it is proposed that the conditions achieved by the illumination of the uptake mutant in the presence of protoporphyrin IX are ideal as a second step in the establishment of full light regulation of the p^{QRS} promoter in E. coli.

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Further, preliminary results indicated that the presence of carotenoid genes cloned from Erwinia herbicola did protect the cell from photosensitization by protoporphyrin IX. This effect requires further study, as does the nature of the photosensitization. However, it does suggest a similar mechanism is seen in the photolysis of M. xanthus and E. coli. It should be cautioned that the possibility remains whereby the mutation which allows the uptake of protoporphyrin IX into the cell, also imparts susceptibility to photolysis. The rationale for this would be that visible-light-mediated porphyrin/cell interactions have been shown to occur predominantly at the membrane (see Introduction for references); mutations allowing uptake of protoporphyrin IX, are most likely to effect the cell membrane. Since both share a common site of action the latter may effect the former. This remains an area open to study.

A second heterologous system was considered for use in this study. Streptomyces species are of considerable industrial potential. Promoter probe plasmids containing the p^{QRS} promoter were constructed and introduced into Streptomyces lividans (results not shown). However, the technical problems experienced with E. coli prompted a postponement in the commencement of full expression studies.

Expression of the p^{QRS} promoter in M. xanthus was studied using a promoter-less lacZ gene in the strain DK101[pDAH217]. Previous results (Hodgson, (1987)) had shown that light causes an increase in p^{QRS} activity over a dark grown basal level. The increase peaks and then falls to an induced basal level. In this study it has been shown that the decrease in p^{QRS} activity occurs as carotenoids begin to accumulate maximally within the cell. In the absence of endogenous carotenoids p^{QRS} activity was not decreased to the same extent as the wild type strain. The decrease in the

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rate of increase in promoter activity seen in the absence of carotenoids probably represents the point at which maximum rate of expression under the conditions used can no longer be maintained. This point may be determined by a limiting step in some metabolic process. It was not possible to determine the light induction profile in the presence a large quantity of exogenous carotenoid. The expected result would be that carotenoid would quench the light signal, as is seen in the presence of the singlet oxygen quencher, DABCO, thus p^{QRS} activity would remain at a very low level. An analogous experiment was carried out using oxygen as the inducer of the p^{QRS} promoter. As discussed in Chapter 5, it was shown that oxygen can induce promoter activity. A steady state culture was exposed to white light at low oxygen tensions. This resulted in an increase in p^{QRS} activity and the accumulation of carotenoids. Increasing the oxygen tension while maintaining the light stimulus did not result in an increase in p^{QRS} activity. This may be interpreted in either of two ways: 1) the carotenoids synthesised de novo, quench the increased "signal" with efficiency sufficient to negate further induction of the promoter; or 2) the promoter is induced at a maximal level and further stimulus cannot yield further induction due to some limiting metabolic process. It may be possible to disregard the latter possibility since experiments in this study and elsewhere have shown p^{QRS} levels considerably higher than those achieved in the continuous culture can be achieved. In particular the Car^C mutant DK408, containing the mutation carR4 shows levels of p^{QRS} activity 10000 times higher than the basal dark grown level of expression (D.Hodgson pers. comm.). However, it should be noted that all other experiments utilize batch culture, which may not allow direct comparison to continuous culture.

Conclusions.

It has been shown that induction of p^{QRS} activity in identically grown batch cultures at differing light intensities proceeds in a linear fashion, increasing with increasing light intensity between 35-550 microE $m^{-2} s^{-1}$. This may be interpreted to show that between these two values only one photoreceptor is involved in the primary photoreceptive event. It should be noted that the possibility does exist that a second photoreceptor may be involved outside the range examined, and that a second photoreceptor of very low extinction coefficient may contribute to the photoreception within the range studied.

A marked decrease in p^{QRS} expression during identical light stimulation was noted as the dark grown culture proceeded through its growth cycle. The reason for this effect may be that the cells from a lag phase culture physiologically stressed, and are beginning to adapt to these stresses. This in general is seen as a slowed metabolism and thus may diminish the capacity of the cells to respond to a light signal. The decreased p^{QRS} activity seen in lag phase cultures is in agreement with the observation of Burchard and Dworkin (1986), who showed that lag phase cells were photosensitive where log phase cells were not. They attributed this difference to the increased accumulation of protoporphyrin IX in lag phase cells. If this were responsible it would be expected that p^{QRS} induction would be maximal in cells from a lag phase culture.

The action spectrum produced by Burchard and Hendricks (1989) showed that a porphyrin molecule was the likely photoreceptor/sensitizer in carotenogenesis/photolysis. They subsequently identified the molecule as being protoporphyrin IX. The presence of this molecule in the strain DK101 was confirmed in this study. The action spectrum of carotenogenesis was determined by measuring the accumulation of carotenoid pigment, when

exposed to different wavelengths of light. This represents the end-product of the induction. To determine if the same action spectrum applied to the induction of the p^{QRS} promoter, a number of experiments were carried out using defined wavelengths of light. It appears that the failure of numerous attempts to induce the promoter may be attributable to low light intensity, since laser light of high intensity, and very defined wavelength, resulted in a massive induction. This demonstrated the promoter may be induced using light at 410nm, which forms part of the characteristic Soret region which all porphyrins exhibit. Further analysis to determine p^{QRS} induction by other wavelengths is required before other molecules such as carotenoids and flavins may be excluded as photoreceptors in the primary photoinductive process.

The mechanism by which the photoreceptor induces expression from the p^{QRS} promoter was examined under steady state continuous culture and in batch culture. The absolute requirement for oxygen in the inductive process could not be determined by studying light induction in the absence of oxygen since M. xanthus is an obligate aerobe. The production of wild-type promoter expression in E. coli would have facilitated this determination since E. coli is a facultative aerobe. In the absence of such a system, it was decided to study the effect of different steady state oxygen tensions on the induction of the p^{QRS} promoter in both the light and the dark.

Steady state cultures of M. xanthus grown under low oxygen tension showed a significant increase in promoter activity induced by an increase in oxygenation. This result was seen in cultures grown with a doubling time of 20 or 10 hours. A similar result was seen under steady states at three progressively increasing oxygen tensions, which resulted in increasing p^{QRS}

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activities. This proves that the need for light to induce the promoter may be circumvented by the addition of oxygen alone. In addition the nature of the interaction between the photoreceptor/sensitizer, protoporphyrin IX, and the signal to the p^{QRS} promoter, presumed to involve the carR gene product, is shown to be a type II interaction, i.e. the signal is provided by a reactive oxygen species. There were two possible interactions envisaged to occur between the photoreceptor and the CarR protein. Firstly, the protoporphyrin IX molecule may be closely associated with the CarR protein, photoexcitation leads to a conformational change or charge transfer which results in the signal being transmitted to induce the p^{QRS} promoter. Secondly, the protoporphyrin IX molecule is "remote" from the CarR protein. Photoexcitation of the porphyrin molecule results in a triplet state, as previously described, which may interact with oxygen to form reactive species. These reactive species diffuse and interact with the CarR protein resulting in the activation of the p^{QRS} promoter. The fact that oxygen alone, a diffusible species, can cause stimulation of the promoter suggests that the primary photoinductive effect is a type II reaction.

The continuous culture with a growth rate of 0.035hr^{-1} did not show a large increase in p^{QRS} activity on exposure to light. This may be attributed to the poor physiology of the slower growing cells, or perhaps the slower growth rate yields a slower response which is sufficient to protect the cell but does not achieve the rapid peak seen in faster growing cells. Continuous culture with a growth rate of 0.089hr^{-1} was used to study the effect of light and oxygen on p^{QRS} expression. It was discovered that the level of oxygenation determined the extent of induction of the p^{QRS} promoter by light. Under limiting oxygen tensions the initial increase in p^{QRS} activity decayed rapidly to a basal level almost equivalent to that

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seen in a dark-grown un-induced culture. Increasing oxygen tension results in an increase in the peak of activity immediately post-induction and in the elevated basal induced level of expression from the p^{QRS} promoter which is established after the initial peak in activity.

Under conditions of steady state continuous illumination, increasing the oxygen tension did not result in an increase in p^{QRS} activity. The most likely explanation for this observation is that the basal synthesis of carotenoids under the initial conditions is sufficient to quench the increased induction signal such that despite the increase in photolytic potential the photodynamic action remains constant and thus does not affect expression from the promoter. The latter case may be envisaged if the basal expression established by light induction at the lower oxygen tension is sufficient to produce sufficient carotenoid to saturate the membrane, or more specifically the sites in the membrane at which protoporphyrin IX acts. It may be that under conditions of steady state continuous illumination, the incorporation of further carotenoid into the membrane is not possible, or the signal to the promoter cannot be propagated beyond the level seen in the membrane at the point of saturation. It is apparent that in a culture of dividing cells a complete cessation in carotenoid synthesis will not be seen since the carotenoids effectively lost in division, or in a continuous culture, in wash out, will have to be replenished to maintain the level of photoprotection required under the inductive conditions applied.

A second effect of oxygen on carotenogenesis was noted. The increase in oxygen tension even when not accompanied by an increase in p^{QRS} activity led to an increased accumulation of a dark red pigment(s). This red pigment(s) could not be identified in preliminary experiments.

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however, it was noted that the increase in red pigment was accompanied by a concurrent decrease in the complexity of the carotenoids developed on a HPLC. It is likely that the increase in oxygen is acting in two possible ways to cause the observed phenotype. Firstly, it may be that oxygen is a limiting factor in the synthesis of the red pigment(s). As such increasing the oxygen tension allows the incorporation of some oxygen requiring group into the carotenoid structure at a greater rate. Secondly, oxygen may activate the expression of a specific oxidase responsible for the incorporation of an oxygen molecule/atom into the carotenoid structure. The nature of the red pigment(s) accumulated and the method of stimulation by oxygen, remain to be elucidated, however, it may be concluded that this effect occurs in addition to, and is separate from, the accumulation of carotenoids produced by the activation of the p^{QRS} promoter.

Having established that oxygen effects the activity of the p^{QRS} promoter, i.e. that the induction of carotenogenesis is a photooxidative event, the nature of the activated oxygen species involved was studied. Bacterial lawns and batch cultures were used to allow more rapid experimentation than is possible with continuous culture.

Addition of hydrogen peroxide, and methyl viologen to a dark grown culture did not induce an increase in p^{QRS} activity, above that routinely seen in a dark grown culture. This suggests that neither hydrogen peroxide nor the superoxide radical were involved in the induction of the p^{QRS} promoter. Heat shock had very little effect on expression from the p^{QRS} promoter. This is in agreement with the hydrogen peroxide result since it is known that hydrogen peroxide is a potent inducer of the heat shock stress response.

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DABCO, a specific quencher of singlet oxygen species, was shown to reduce light activation of p^{QRS} . This reduction in p^{QRS} activity occurred independently to any effects on the growth of the culture. This suggests that the oxygen species involved in the activation of the promoter is singlet oxygen.

To confirm the role of singlet oxygen in the induction of p^{QRS} , a specific singlet oxygen producing photosensitizer, toluidine blue, was used to induce the promoter. It was shown that in a culture of M. xanthus induced by white light, the activation of the p^{QRS} promoter is stimulated by the presence of toluidine blue. Thus indicating that toluidine blue was capable of influencing promoter expression in some manner. It was shown that the activation of p^{QRS} by white light in the presence of toluidine blue may be quenched by the addition of DABCO. Thus showing both sensitizers are acting through the singlet oxygen molecule. However, the possibility exists that toluidine blue is acting in some way through the native photoreceptor, protoporphyrin IX. To dissect the effects of toluidine blue from the effects of protoporphyrin IX induction of the p^{QRS} promoter by red light was studied. It was shown that red light in the absence of toluidine blue was unable to induce p^{QRS} activity. However, in the presence of toluidine blue a large increase in promoter activity was seen in cells which were irradiated with red light. It was subsequently shown that this induction could be specifically quenched by the addition of DABCO. This provided the final proof that toluidine blue could specifically activate the p^{QRS} promoter, in the absence of induction by the native photosensitizer, by the production of singlet oxygen.

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7.2. Future approaches in the furtherance of this study.

The use of E. coli as a heterologous system, still remains the most logical approach as a forerunner to the use of the p^{QRS} promoter in other potential industrially important strains. Future studies employing E. coli should concentrate on utilizing the 0.4 kb, Sac I/Bgl II fragment of the promoter region. This fragment appears most likely to express the true p^{QRS} promoter, and is not affected by what appears to be a second promoter region upstream of the p^{QRS} promoter. Preliminary results appeared to show that the p^{QRS} promoter could be stimulated by a product of the carQRS region. This stimulation being dependent on the orientation of the region. This stimulation may be investigated further by a number of approaches. It should be determined which if any of the orfs present in the carQRS region are responsible for this stimulation. This may be done by the use of a number of mutants isolated and characterized as occurring within the carQ, carR, and carS genes. These mutants have been isolated and may be introduced into E. coli using routine techniques. It should be determined that the stimulation is occurring via a factor acting in trans. This may be done by the use of plasmids of differing incompatibilities. One plasmid carrying the 0.4kb promoter region, the second carrying the carQRS region.

It has been established that the E. coli strain which exhibits enhanced uptake of protoporphyrin IX is susceptible to photolysis. The p^{QRS} promoter is induced by the products of photolysis and so the uptake mutant remains a useful tool to study the absolute requirements for the induction process. A prerequisite to its use would be that all regions of DNA which could play a potential role in the inductive process should be

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cloned into the E. coli host. Preliminary experiments will be required to determine the light intensity and protoporphyrin IX concentration at which the culture is stressed by the light signal but not lysed. If all required genetic elements are present the light signal should have the effect of inducing high levels p^{QRS} expression. It may be interesting to place the carotenoid genes, cloned from Erwinia, under the control of the p^{QRS} promoter in an attempt to obtain a true induction in which a constant induced level of p^{QRS} expression is seen.

With regards to the study of p^{QRS} expression in M. xanthus, there are two areas of research initiated in this study which require further analysis. The first is in the identification of the carotenoids whose accumulation is promoted in a steady state, illuminated, continuous culture by increasing oxygenation of the culture. It is most likely that the pigments are myxobactin, and myxobacton, however, this needs to be confirmed. The second is in the production of an action spectrum of the induction of the p^{QRS} promoter. It has been shown that using the experimental design employed, high light intensities of defined wavelength light are required to obtain a measurable result. It appears intensities of at least 50-80 microE m⁻² s⁻¹ will be required. Such intensities may not be possible using conventional light sources, and may require laser light. Such experiments are not at present practical, which may result in a change in the experimental design being necessary. It may be possible to obtain sufficiently consistent results from M. xanthus cultured on solid media to allow an identical experimental design to that conducted by Burchard and Hendricks (1989).

With the identification of the sequence of a number of genes involved in the induction of p^{QRS} it is now possible to analyse their interaction and

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the precise times at which these genes are expressed, post light induction. Such analysis may be performed using specific DNA probes and standard techniques, such as Northern transfer of mRNA, and dot blots. The time at which transcription from p^{QRS} is begun may be more accurately determined by probing for the presence of mRNA encoded by the lacZ gene using a gene specific DNA probe.

The various stages at which protein synthesis is required may be studied using a protein synthesis inhibitor, such as chloramphenicol. Isolation of the proteins and the subsequent production of antibodies will allow specific studies of the regions of the cell in which the protein is found, and also the level at which the protein is regulated, i.e. at transcription or translation. Similarly, it is possible that the proteins involved in the induction of p^{QRS} are subject to degradation by light, and it may be possible to follow the light promoted destruction of the translated protein, using antibodies to that protein.

It is apparent from the studies utilizing the singlet oxygen quencher DABCO that exposure to low concentrations after a period of some quenching results in the promotion of p^{QRS} expression, to levels above that which is seen in a culture lacking DABCO (Chapter 6.3.1. and 6.4.2.). One possible explanation of this observation would be that low levels of quenching promote the accumulation of some factor whilst preventing the synthesis of carotenoid. The lack of synthesis of carotenoid allows the build up of this factor to levels which are not normally achieved during normal light induction. Thus when the level of quenching is reduced the response seen is considerably greater than that achieved under normal conditions. The level of quenching may be reduced by one or a combination of two possible mechanisms: The quencher, DABCO, may be subject to

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photodestruction, or the increase in the number of cells in the culture dilutes the concentration of quencher per cell beyond a critical level, below which quenching is no longer seen. The factor which is accumulating under these conditions may be investigated by either of the above techniques; mRNA analysis using specific DNA probes, or protein analysis using antibodies.

It has been shown in this study that singlet oxygen is the light generated inducer of p^{QRS} expression by the native photoreceptor, protoporphyrin IX. In addition, it has been shown that an exogenous photopigment, toluidine blue may substitute for the native photoreceptor, and induce the p^{QRS} promoter via the generation of singlet oxygen. Should further proof of the activation of p^{QRS} by singlet oxygen the method of Dahl (1987) may be employed. In this method the cells are separated from the inducer by a layer of air through which only singlet oxygen may diffuse. The layer forms a barrier to oxygen radicals, and the separation of photosensitizer and cell eliminates the possibility of a direct interaction of the photosensitizer and the signalling molecule, presumed to be the CarR protein. Further analysis may be carried out using the large number of quenchers and photochemicals available to the study of photochemistry. Such a broad approach would remove the possible criticism that effects seen with a specific chemical in M. xanthus may not necessarily be of a similar nature to the effects seen in other systems, especially in vitro.

Having established the molecular species involved in the primary photochemical event, the next most logical area of research would be to determine how the molecular species generated by light interacts with the signalling protein, presumed to be the product of the carR gene. It has been shown that singlet oxygen specifically targets five amino acid

residues. These residues are cysteine, histidine, methionine, tryptophan, and tyrosine. There are twenty four of these residues on the CarR protein, seventeen of which occur in the proposed trans-membrane region, which provide an initial target for site directed mutagenesis. Conversion of the target residues to residues which singlet oxygen cannot target would hopefully remove the sensitivity of the CarR protein to singlet oxygen, thus producing a mutant in carR which expresses a Car⁻ phenotype, such a mutant has not previously been isolated.

APPENDIX.

Appendix A. Computer program to calculate beta-galactosidase in M. xanthus.

This program is written to calculate only beta-galactosidase activity according to the protocol outlined in Methods 2.3.2. The program is written to run on Mallard BASIC available to the Amstrad PC9512. The program utilizes raw data as determined experimentally, except for protein values; these must be converted to micro-g according to the standard curve obtained.

A brief title, not exceeding one line in length may be entered at the start of the calculations. Zero values may be entered for ONPG optical densities, but not for protein values. Should an error occur when inputting data, a function at the end of the input sequence allows the operator to restart, erasing only those values input for the sample currently being calculated. The order of the input lines may be altered at the programming level to allow for different data formats; however, care must be taken to adjust any GOTO functions that may be altered. There are no limits to the number of samples which may be calculated, simply answer "Y" to the question "are there any more calculations". Different titles may be introduced during the calculations by answering "N" to the question "are they from the same experiment".

The values are output to two decimal places and include the values from which the final value was calculated for error analysis.

Note: Ease of input is facilitated using the right hand grouped number keys which are obtained on the Amstrad 9512 via the "ALT", "SHIFT", "RELAY" option.

```

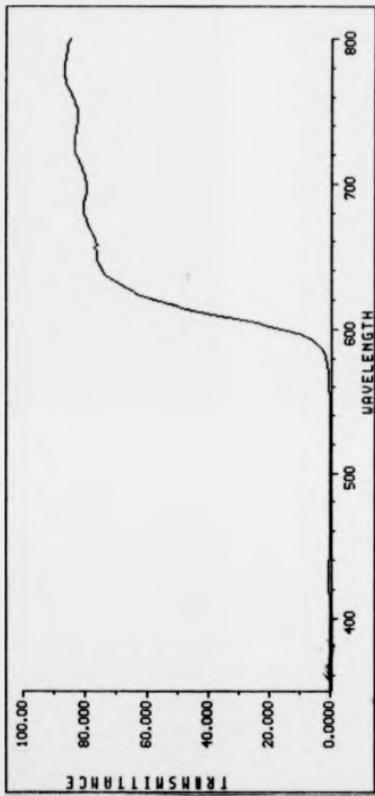
10 GOTO 40
20 LPRINT ""
30 PRINT "enter new title."
40 WIDTH LPRINT 70
50 INPUT "enter experiment title. "; title$
60 LPRINT title$
70 INPUT "enter subtitle if required. "; title1$
80 LPRINT title1$
90 LPRINT ""
100 LPRINT ""
110 GOTO 130
120 PRINT "enter all values again."
130 PRINT ""
140 INPUT "enter the culture timepoint. "; timept
150 INPUT "enter largest vol. of cells used in beta-gal assay. "; betavo
160 INPUT "enter the smaller vol. of cells used in beta-gal assay. "; be
    tavol2
170 INPUT "enter the no ONPG blank for this volume"; onpg0
180 INPUT "enter first ONPG O.D. for the larger sample. "; onpg1
190 INPUT "enter the second ONPG O.D. for the larger sample. "; onpg2
200 INPUT "enter first ONPG O.D. for the smaller sample. "; onpg3
210 INPUT "enter second ONPG O.D. for the smaller sample. "; onpg4
220 INPUT "enter the incubation time for the large sample (mins). "; tim
    e1
230 INPUT "enter the incubation time for the small sample (mins). "; tim
    e2
240 INPUT "enter the 5 micro-l protein determination. "; prot1
250 INPUT "enter the 10 micro-l protein determination. "; prot2
260 INPUT "are these values correct (y/n)"; answer$
270 GOTO 290
280 INPUT "Y or N"; answer$
290 IF answer$="n" GOTO 120
300 IF answer$="y" GOTO 320
310 GOTO 280
320 LET protav=(prot1+prot2) / 2
330 IF onpg2=0 GOTO 360
340 LET corronpg1=((onpg1+onpg2)/2) - onpg0
350 GOTO 370
360 LET corronpg1= onpg1 - onpg0
370 IF onpg4=0 GOTO 400
380 LET corronpg2=((onpg3+onpg4)/2) - (onpg0*(betavol2/betavol1))
390 GOTO 410
400 LET corronpg2= onpg3 - (onpg0*(betavol2/betavol1))
410 LET beta1=((corronpg1/protav) * (7.5/betavol1) * (233.33333f/time1
    )) * 1000
420 LET beta2= ROUND(beta1,2)
430 LET beta3=((corronpg2/protav) * (7.5/betavol2) * (233.33333f/time2
    )) * 1000
440 LET beta4= ROUND(beta3,2)
450 LET betafin=(beta1+beta3) / 2
460 LET betafin2= ROUND(betafin,2)
470 LPRINT "Beta-gal activity for"; timept "hr(s). is", betafin2
480 LPRINT "the"; betavol1 "micro-l value="; beta2", the"; betavol2 "micro
    -l value="; beta4
490 LPRINT ""

```

```
500 INPUT "Are there any more calculations? (Y/N)";answer2$
510 IF answer2$="n" GOTO 580
520 IF answer2$="y" GOTO 540
530 GOTO 500
540 INPUT "Are they from the same experiment? (Y/N)";answer3$
550 IF answer3$="n" GOTO 20
560 IF answer3$="y" GOTO 130
570 GOTO 540
580 PRINT "CALCULATIONS COMPLETED."
590 END
```

Appendix B. Transmission spectrum of red filter:

The percentage of light transmitted over a range of wavelengths by the red filter used in experiments 6.4.1., and 6.4.2. is shown.



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