Further Genetic Studies of the carQRS Region of *Myxococcus xanthus*.

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NUMEROUS ORIGINALS
IN COLOUR

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Declaration:

The work described in this thesis is the result of original research conducted by myself under the supervision of Dr. D.A. Hodgson. In the instances where others have contributed to the work, specific acknowledgements have been made.

None of the information contained in this thesis has been used in any previous application for a degree.

Simon McGowan.

Summary:

The light-inducible carotenogenic response of Myxococcus xanthus has previously been shown to be controlled by the three genes of the carQRS operon. We describe a function for each of these three genes, and a possible mechanism by which they act in concert to carry out those functions.

The promoter of the three genes, previously described as light-inducible, is negatively controlled by the product of the carR gene. We show that this negative control acts through the product of the carQ gene, itself positively required for promoter activity.

We present a model for the light-induction of the promoter of the carQRS operon (P^{QRS}). In the dark, the promoter-activating, carQ gene product is sequestered to the membrane by the product of the carR gene. Upon light-induction this sequestration ceases and the CarQ protein is released to cause increased expression of the carQRS operon. There is evidence that, upon light-induction, the CarR protein is actively degraded by a protease, possibly encoded by the carD gene. There is an absolute requirement for the maintenance of translational coupling between the two genes, carQ and carR. The model is extended to show how translational coupling could aid the sequestration of CarQ to the membrane.

Only some of the structural genes (those at carB) are activated by the carS gene product. CarS is not required for the activation of P^{QRS}. The structural genes at carC are shown to be activated by the carQ gene product.

A preliminary analysis of the structure of P^{QRS} is presented. There appears to be a functional requirement by this promoter for an intact promoter of the upstream and divergently transcribed gene, sufA.

An extension to the DNA sequence of the chromosome adjacent to the carQRS operon is presented.

List of abbreviations:

A	absorbance
allolactose	6-O-β-D-galactopyranosyl-D-glucose
Ap	ampicillin
bp	base pair(s)
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dITP	2'-deoxyinosine 5'-triphosphate
DNA	deoxyribonucleic acid
dTTP	2'-deoxythymidine 5'-triphosphate
E. coli	Escherichia coli
EDTA	diaminoethanetetra-acetic acid
IPTG	isopropyl β-D-thiogalactopyranoside
Kb	kilo base pair(s)
Km	kanamycin
light-inducible promoter	the promoter of transcription of carQRS (also termed, p^{QRS})
M. xanthus	Myxococcus xanthus
OD	optical density
ONPG	O-nitro-phenol-β-D-galactose pyranoside
pfu	plaque forming unit(s)
p^{QRS}	the promoter of transcription of carQRS (also termed, the light-inducible promoter)
SDS	sodium dodecyl sulphate
Tris	2-amino-2(hydroxymethyl)-1,3-propane diol
UV	ultra violet
XGAL	5-bromo-4-chloro-3-indolyl-galactoside

Chapter 1.

Introduction.

1.1 Myxobacteria:

Myxobacteria, discovered exactly 100 years ago (Thaxter, 1892), live in soils throughout the world, where they feed upon organic matter and prey upon other microorganisms. They have an unusual life style, existing as either single cells or as multicellular units. At times of starvation, cells aggregate to form compact structures known as fruiting bodies. Within these structures, containing around 100,000 cells, are formed UV, freezing, heat and desiccation resistant myxospores (reviewed by Kaiser, 1988).

Myxobacteria move by a process known as gliding. This form of locomotion is widely distributed throughout the eubacteria and appears not to be phylogenetically related (Ludwig et al., 1983). Myxococcus xanthus has two distinct genetic systems for the control of gliding; social and adventurous (reviewed by Burchard, 1984). A cell with a mutation in the A system can no longer move independently of other cells, whereas a mutation in the S system leads to movement by single cells only. A strain that is S^-, therefore, is incapable of forming fruiting bodies. Only one locus, the mgl locus, is common to both systems and would appear to be directly involved in the as yet uncharacterised gliding mechanism.

Many years ago, the micropredatory life style of the myxobacteria was recognised as making them ideal candidates for the production of antibiotics (Singh, 1947). Since that time, a wide range of such compounds have been isolated and their structure realised (for a review, see Reichenbach et al., 1988). It is believed that around 80% of myxobacteria produce antibiotics (Foster et al., 1982). As was pointed out by Reichenbach et al., (1988), however, although novel antibiotics undoubtedly remain to be discovered, because of their relatively narrow phylogenetic diversity, the

myxobacteria will probably never be as rich a source of these compounds as the actinomycetes.

Myxobacteria are Gram-negative organisms and have been shown by 16S rRNA analysis to be members of the δ-subdivision of the purple bacteria (Oyaizu and Woese, 1985; Devereux *et al.*, 1990). Within the myxobacteria, the existence of three separate groups has been recognised, although there is disagreement about their relative nomenclature (Ludwig *et al.*, 1983; Shimkets, 1990). There is agreement, however, over the sub-order Cystobacterineae which includes *M. xanthus*, as well as *Stigmatella aurantiaca* and *Cystobacter fuscus*.

The genome of *M. xanthus* is one of the largest bacterial genomes at around 9500 kbp (Chen *et al.*, 1991). Despite this large size, these authors have been able to construct a circular restriction map of the chromosome using pulsed field gel electrophoresis and a yeast artificial chromosome clone library. The base composition of myxobacterial DNA is also relatively high at between 67 and 71 mol% G + C (Mandel and Leadbetter, 1965).

The ability to carry out genetic analysis of *M. xanthus* (reviewed by Kaiser, 1991) is aided by the availability of several myxophages capable of generalised transduction (including the virulent phage Mx4 and the temperate phage Mx8) and the coliphage, P1. The latter is able to adhere to *M. xanthus* and inject its DNA, but cannot then replicate within the cell. This phage permits the introduction of transposons such as Tn*5* and Tn*5* *lac*. No naturally occurring plasmid has been found in *M. xanthus*, although the construction of an artificial plasmid utilising the Mx4 origin of replication has recently been reported (O'Connor *et al.*, 1991).

This study will concentrate upon another property of the myxobacteria not so far described - their ability to produce carotenoids.

1.2 Carotenoids:

Carotenoids are probably the most widely distributed class of pigments found in nature, occurring in plants, animals and microorganisms (Young, 1991). They appear to have a dual role in photosynthetic organisms. They may act as accessory light-harvesting pigments and thereby extend the spectral range available to the photosynthetic apparatus (Frank et al., 1981).

Their second role is of more relevance to this study. Carotenoids have been shown to "photoprotect" the cell from light-mediated stress (Griffiths et al., 1955). Plants that have been treated with herbicides to prevent the formation of carotenoids, for example, are quickly killed when exposed to light (Moreland, 1980).

1.2.1 The photoprotective effect of carotenoids:

Rather than simply blocking the initial activating photon of light, carotenoids achieve photoprotection primarily by quenching triplet state chlorophyll molecules. They also quench singlet oxygen and may scavenge free radical intermediates generated by the activated chlorophyll. The terms "triplet" and "singlet" refer to the configuration of electrons within the molecule. A molecule is said to be in the ground state if electrons within that molecule occupy paired, low energy orbitals and have antiparallel spin. If the molecule becomes photo-activated and one of these electrons captures the energy of excitation and is promoted to a higher energy orbital, the molecule is said to be in the excited triplet state. If that electron subsequently undergoes "intersystem crossover" to reverse its

direction of spin, the molecule is now said to be in the singlet state.

Molecular oxygen is unusual because it is in the triplet state and is very stable. Excitation of this molecule by energy transfer from a photo-activated species (the photosensitiser - in this case triplet chlorophyll), however, leads to the formation of the far more reactive singlet oxygen. This species is a powerful oxidising agent and, if unquenched (i.e. the energy of excitation is retained), will cause damage to the cell by reaction with DNA, ribosomes (Singh et al., 1984), proteins, membranes and chlorophyll itself.

In quenching photoactivated chlorophyll, carotenoids have been shown to reduce the lifetime of this triplet state by three orders of magnitude (Cogdell, 1985). In the absence of oxygen, this prevents the formation of the hydroxyl and other free radicals that may form in redox reactions between the activated photosensitiser and various cellular components.

In the presence of oxygen, quenching reduces the time available for any reaction between the oxygen and the photosensitiser, and thus effectively prevents the formation of the damaging singlet oxygen species. Carotenoids are also able to interact with singlet oxygen to quench it directly (Di Mascio et al., 1989), although this particular reaction remains poorly understood. Following quenching, the now activated carotenoid molecule decays back to its ground state with the dissipation of energy in the form of harmless heat.

In order to be efficient in quenching, the carotenoids must have a lower triplet energy state than the energy level of the photoactivated species. In order to gain this low energy state to quench, for example singlet oxygen, the carotenoid molecule must have between seven and nine conjugated double bonds (Mathews-Roth et al., 1974; Cogdell, 1988). In

addition, work involving Chlorella and the photobleaching of chlorophyll in the absence of oxygen, has shown a minimum of seven conjugated double bonds are required to quench a triplet state photosensitiser (Rau, 1988).

1.2.2 Bacterial photolysis:

Photolysis, the killing of a cell by a light-activated chemical compound in the presence of molecular oxygen, has been demonstrated in a wide range of bacteria. Work in 1956 confirmed that carotenoids could protect the photosynthetic bacterium Rhodopseudomonas spheroides (now renamed Rhodobacter spheroides) from the deleterious effects of light (Sistrom et al., 1956). Later reports have indicated similar photoprotection in carotenoid producing but non-photosynthetic bacteria. These include Sarcinia lutea (Mathews and Sistrom, 1959), Micrococcus roseus (Dieringer et al., 1977) and Bdellovibrio bacteriovorus (Friedberg, 1977). In addition, carotenoids have also been shown to offer specific protection against singlet oxygen in at least three separate species, Staphylococcus aureus, Sarcinia lutea (Dahl et al., 1988) and Rhodospirillum rubrum (Boucher et al., 1977).

Photolysis has been demonstrated in bacteria that do not normally produce carotenoids, such as Escherichia coli. Tuveson et al., (1988) have cloned and expressed the genes responsible for the production of carotenoids from Erwinia herbicola in E. coli. The carotenoids produced were shown to prevent photolysis by near-UV light (320 - 400nm) as well as by visible light in the presence of various photosensitising chemical species.

Of particular relevance to this study, is the observation that a

mutation within the visA/hemH gene at around 11 minutes on the E. coli chromosome, causes a lethal sensitivity to visible light (Miyamoto et al., 1991). The authors speculate that it is the accumulation of protoporphyrin IX caused by this mutation that leads to the observed phenotype. As described below, protoporphyrin IX is thought to be the photosensitiser present within Myxococcus xanthus.

1.2.3 Photolysis and Myxococcus xanthus:

Photolysis of Myxococcus xanthus was first demonstrated in 1966 (Burchard and Dworkin, 1966). An analysis of the action spectrum involved in this process (previous ref. and Burchard et al., 1966) indicated that protoporphyrin IX was the likely photosensitiser. Photolysis could only be induced in cells that were in the stationary phase of growth. This correlated with a 16-fold increase in the amount of protoporphyrin found within the cells during this phase of growth. The authors speculate that the protoporphyrin is either a precursor or breakdown product of cytochromes.

In the dark M. xanthus is yellow. Cells exposed to light in the logarithmic phase of growth however, produce orange, light-protective carotenoids. These carotenoids are only produced in response to light and in this way the bacterium avoids the energy requiring process of carotenogenesis in the absence of light-mediated stress. Carotenogenesis in M. xanthus was characterised and found to be a blue light (405 - 410nm) requiring process (Burchard and Hendricks, 1969). In addition, the action spectra of carotenogenesis and light-induced photolysis were found to be

very similar and protoporphyrin IX was, therefore, proposed to be the photosensitiser involved in both processes.

1.3 Light as an environmental signal:

1.3.1 Photoregulated plant gene expression:

Light has been used by a wide range of organisms to regulate many different cellular processes. Nowhere is this more striking than in higher plants where, for example, the formation of chlorophyll and the small subunit of RuBisCO, as well as the opening of stomata are all dependent upon light. The best characterised of the photoreceptors known to be used by plants is phytochrome. This pigment absorbs light in the red/far-red portion of the electromagnetic spectrum, whereas other prominent plant photoreceptors respond to blue light and UV-B light.

Little is known, however, about events occurring after activation of the photoreceptor. Although evidence has been presented for control by light at the post-transcriptional level, most work in this area has concentrated on the control of gene expression (Ellis, 1986; Fluhr *et al.*, 1986). Factors such as different expression patterns of genes activated by the same photoreceptor and evidence for multiple control sites upstream of these genes (reviewed by Gilmartin *et al.*, 1990), would seem to argue for a greater level of complexity than that envisaged for control of carotenogenesis in *M. xanthus* (see model outlined below).

1.3.2 The light responses of microorganisms:

Some cyanobacteria are able to alter the various components of the phycobilisome, the major light harvesting complex, in response to both the quantity and quality of light (Oelmuller et al., 1986). This process, known as complementary chromatic adaption, allows the organism to make the best use of incident light (for example, at different depths in water). Regulation at the level of transcription in response to red or green light causes increased production of either phycocyanin or phycoerythrin respectively. Unlike the myxobacterial carotenogenic response, however, only short pulses of light are required to initiate synthesis of these proteins in subsequent darkness. Although the genes encoding these proteins have been cloned and sequenced, the mechanism of control of their light-inducible expression remains unclear.

Another well studied photosensory response of motile microorganisms is the movement towards or away from a light source (phototaxis). Again, although the action spectra and photoreceptors may be analysed and the type of movement with respect to the light characterised, little is known about how the light signal regulates the motility apparatus (reviewed by Hader, 1987).

In conclusion, although at first, light responses in other organisms would seem to be related to the light-inducible production of carotenoids by Myxococcus xanthus, in reality this is probably not the case. Intriguingly however, some similarities can be found. These include the response to blue light by the plant pigment, cryptochrome and the apparent involvement of singlet oxygen (see section 1.7.1) in the phototactic response of the cyanobacterium, Anabaena variabilis (Schuchart and Nultsch, 1984).

1.3.3 Myxobacterial light responses:

As described above, myxobacteria produce carotenoids in response to light. Light would also appear to have a role in the control of other functions within this group. Certain species of myxobacteria for example, have been shown to be negatively phototactic when moving as a swarm, although not as single cells (Aschner and Chorin-Kirsh, 1970).

Blue light has also been reported to be required for the efficient formation of fruiting bodies in the myxobacterium, Stigmatella aurantiaca (Qualls et al., 1978). Further work has suggested that the light acts to sensitise the cell to the presence of a pheromone required for development (Stephens, 1986).

1.4 The carotenoids of myxobacteria:

Several approaches have been taken in trying to understand the light-inducible carotenogenic response of the myxobacteria. One such approach has been to isolate and identify as many as possible of the carotenoids produced by representative members of the different genera.

Although a wide spectrum of carotenoids are produced by the myxobacteria, all are variations of a basic C_{40} chain (Reichenbach and Kleinig, 1984). This variation may take the form of, for example, cyclisation of one end of the chain to form a ß-carotene ring, addition of hydroxyl or keto groups to the chain, or variations in the degree of saturation. The hydroxyl group of the tertiary carbon atom may also be substituted with a sugar molecule, often glucose. In such cases, all have been found to be esterified with one of a variety of fatty acids.

Wide variations in the pattern of carotenoids between the three "groups" of myxobacteria have been found. There appears to be little variation, however, within the Cystobacterineae sub-order to which M. xanthus belongs. Although the pattern of carotenoids found in M. xanthus has not been published, it is believed to be similar to that found in other members of the Cystobacterineae (Reichenbach H. pers. comm.); namely Stigmatella aurantiaca (Kleinig and Reichenbach, 1969, Kleinig et al., 1970) and in particular, M. fulvus (Reichenbach and Kleinig, 1971).

Some 50 to 60 different carotenoids were reported in M. fulvus, of which the 24 most abundant were identified. Only three of these, however, formed by far the majority of the carotenoids observed (Figure 1.1). The blood red pigment, myxobacton ester made up 70% of the extracted carotenoids. The other two major carotenoid species were each found to make up approximately 10% of the total. These two compounds were the red-violet pigment, 4-keto-torulene and the bright orange myxobactin ester.

The authors were able to estimate the amount of carotenoids produced under different growth regimes as a proportion of total dry cell weight. Thus, in the dark, carotenoids made up 0.003% of dry weight. In the light, carotenoids made up 0.03% of dry weight of exponentially growing cells and by late log phase, this had risen to 0.06%.

1.5 The carotenoid biosynthetic pathway:

A second approach adopted in trying to characterise the carotenogenic response of myxobacteria, has been to dissect the chemical pathway of their synthesis with the use of various chemical inhibitors.

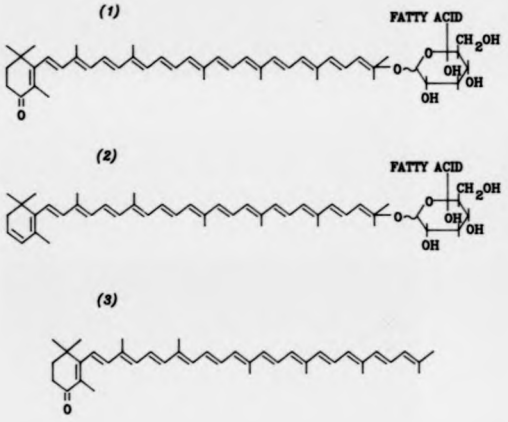


Figure 1.1 The major carotenoids of M. fulvus: The majority of the carotenoids found in this species were (1) the blood red, fatty acid ester of myxobacton (70% of total), (2) the orange, fatty acid ester of myxobactin (10%) and (3) the red-violet, 4-keto-torulene (10%) (Reichenbach and Kleinig, 1971).

In M. fulvus, there are approximately ten steps in the conversion of phytoene, the colourless, first C_{40} carotenoid precursor, into the ester of myxobacton, the main carotenoid end product (Figure 1.2; Kleinig, 1975). Although these steps have been presented sequentially, there is evidence that a number of the enzymes involved may act at different points along this pathway.

The effect of adding the herbicide, San 6706 (4-chloro-5-(dimethylamino)-2-α,α,α-(trifluoro-m-tolyl)-3(2H)-pyridazinone) was to reversibly prevent the formation of any unsaturated carotenoids, and resulted in the accumulation of phytoene. Dehydrogenation of phytoene through the various intermediates of the Porter-Lincoln series (phytoene → phytofluene → ζ-carotene → neurosporene → lycopene; Porter and Lincoln, 1950) are, therefore, the first steps in this pathway.

In the formation of myxobacton ester, however, M. fulvus appears not to complete this Porter-Lincoln series of desaturation reactions prior to the addition of a terminal hydroxyl group. Thus, the pathway proceeds via 1'-hydroxy-neurosporene (structure 7 of Figure 1.2) and does not form the last intermediate, lycopene (structure 8). One of the final desaturation reactions to form a derivative of lycopene occurs later, and probably only after esterification (Kleinig, 1975). This pathway is not rigid, however, and if the desaturation of phytoene is inhibited, the cell is able to make use of exogenously added lycopene for the synthesis of myxobacton ester.

Hydroxylation of the 1' carbon was confirmed as the next step in the pathway by the addition of high concentrations of either the drug, CPTA (2-(4-chlorophenyl-thio) triethylamine hydrochloride) or nicotine. Inhibition of the hydroxylation of the terminal carbon was achieved, although lycopene was accumulated rather than the expected neurosporene (structure 8).

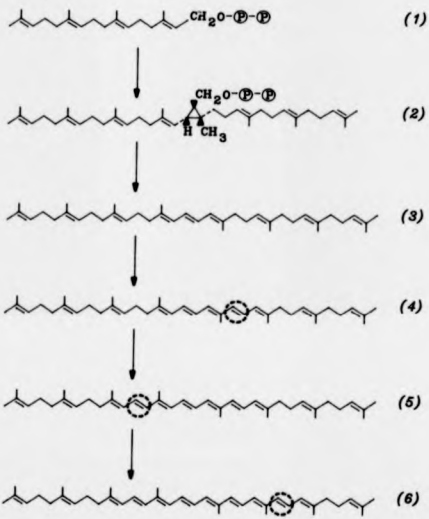
Figure 1.2 The carotenoid biosynthetic pathway of M. fulvus: from geranylgeranyl pyrophosphate to myxobacton ester and 4-keto-torulene: (Adapted from Kleinig, 1975.) Differences in structure between a compound and its preceding intermediate in the pathway have been highlighted in red.

part a: The presence of the proposed intermediate prephytoene pyrophosphate (structure 2) between geranylgeranyl pyrophosphate (structure 1) and phytoene (structure 3) has not yet been demonstrated. The action of the enzyme phytoene dehydrogenase upon phytoene leads to the formation of neurosporene (structure 6), via phytofluene (structure 4) and ζ-carotene (structure 5).

part b: In the formation of the minor product, 4-keto-torulene (structure 10), the last desaturation of the Porter-Lincoln series is carried out to yield lycopene (structure 7) from neurosporene. This compound is then thought to undergo cyclisation and carbonylation before the final dehydrogenation reaction.

part c: In the formation of myxobacton ester (1'-glucosyloxy-3',4'-didehydro-1',2'-dihydro-β,ψ-caroten-4-one ester; structure 15), hydroxylation of the 1' carbon of neurosporene yields 1'-hydroxy-neurosporene (structure 11). This compound then undergoes glucosylation, esterification and dehydrogenation, to form 1'-glucosyloxy-3',4'-didehydro-1',2',7',8'-tetrahydro-ψ,ψ-carotene ester (structure 12). A further dehydrogenation reaction leads to the formation of 1'-glucosyloxy-3',4'-didehydro-1',2'-dihydro-ψ,ψ-carotene ester (structure 13). Cyclisation of this compound yields 1'-glucosyloxy-3',4'-didehydro-1',2'-dihydro-β,ψ-carotene ester (structure 14). Finally, the addition of a keto group to this compound results in the formation of myxobacton ester.

(part a)



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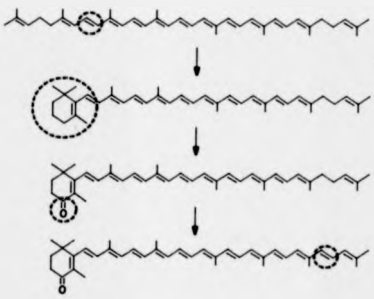

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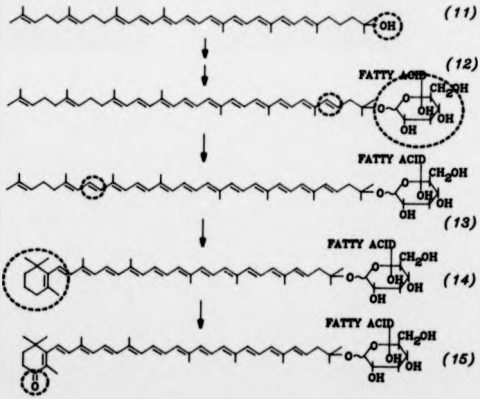


Lower concentrations of nicotine were found to inhibit the cyclisation of the carbon chain and to cause the reversible accumulation of an acyclic carotenoid glucoside ester (structure 13; Kleinig and Reichenbach, 1973; Kleinig, 1974). Thus, glucosidation and subsequent esterification of the carotenoid, occur before cyclisation. If the nicotine was washed off in the absence of molecular oxygen, then unlike the monocyclic carotenoids of aerobically grown cells, a keto group was not added. Carbonylation, therefore, occurs as the last step in the carotenoid biosynthetic pathway after cyclisation.

1.6 The genetics of carotenogenesis:

The third, and arguably most fruitful, approach adopted in the characterisation of carotenoid synthesis, has been to examine the genetics of the process. This has been undertaken in both *M. xanthus* as well as in other carotenoid producing organisms.

1.6.1 The genetics of carotenogenesis in other organisms:

The main thrust of work on carotenogenesis in other organisms has been to characterise the carotenoid pattern produced, elucidate the biosynthetic pathway and, in bacteria at least, identify the genes encoding the enzymes involved in that pathway. The genetics of, for example, the phototrophic bacterium *Rhodobacter capsulatus* involved in the production of the acyclic carotenoid, spheroidene, have been examined. The complete carotenoid biosynthetic gene cluster of this organism, has been sequenced (Armstrong *et al.*, 1989). This region encodes eight genes (*crtA*, *B*, *C*, *D*,

E, F, I, K) of which the functions of the products of seven have been elucidated (the exception being crtK; Baker and Fanestil, 1991).

The carotenoid biosynthetic genes of other organisms have also been cloned and in some cases sequenced. These include Erwinia herbicola (Armstrong et al., 1990; Schnurr et al., 1991) E. uredovora (Misawa et al., 1990), Mycobacterium aurum (Houssaini-Iraqui et al., 1992) and the cyanobacterium, Synechococcus (Chamovitz et al., 1992). Although many different carotenoids are synthesised by these and other organisms, all proceed through common early intermediates, namely phytoene and its dehydrogenated derivatives to neurosporene (Armstrong et al., 1990; Schmidhauser et al., 1990; Dogbo et al., 1988).

Light has been shown to be by far the most common environmental stimulus used in the control of carotenogenesis (reviewed by Harding and Shropshire, 1980). With the exception of work carried out in M. xanthus (detailed below and continued in this study), however, little attempt has been made to fully characterise the mechanism of light regulated gene expression. Certainly in eukaryotes where genetic approaches are more complicated, analyses have often been limited to characterisation of the action spectra involved (reviewed by Rau, 1985).

1.6.2 The genetics of myxobacterial carotenogenesis:

A mutant strain of M. xanthus that synthesised carotenoids in both the light and the dark was reported in the original paper of Burchard and Dworkin (1966). The systematic identification of genes involved in the myxobacterial carotenogenic response, however, was first attempted in 1986 by Martinez-Laborda et al. These authors were able to characterise six

mutant strains in which carotenogenesis had become constitutive (Car^C), by transposon tagging several genetic loci on the *M. xanthus* chromosome. Their work defined two loci which appeared to be involved in either the control or production of carotenoids.

This approach has since been continued and has led to the identification of a number of loci that are crucial to the carotenogenic response. These loci may be divided into two types. Some, such as the unlinked loci *carB* and *carC* (Balsalobre *et al.*, 1987) encode the enzymes involved in the carotenoid biosynthetic pathway. Using Tn*5 lac* (Kroos and Kaiser, 1984), the expression of a promoterless *lacZ* gene was fused to the promoters of genes within these regions. Balsalobre *et al.*, (1987) were subsequently able to show that the expression of these genes was light-inducible.

Two other loci, *carA*, which is linked to *carB*, and *carR* (later renamed the *carORS* region) appeared to be involved in the light-inducible control of expression of both *carB* and *carC*. The *carA* region has been reported to encode a *cis*-acting function which appears only to exert an influence over the gene(s) at *carB* (Balsalobre *et al.*, 1987). There is now doubt, however, about the *cis*-acting nature of this function following evidence that *carA* is still able to function when inserted at a different position on the chromosome (Murillo F., pers. comm.).

The *carORS* region in comparison was immediately recognised as more fundamental to the control of the carotenogenic response. Again using the *carB/lacZ* fusions, Balsalobre *et al.*, (1987) were able to show that mutations within this region resulted in a high level of light-independent expression of both *carB* and *carC*. This result seemed to indicate that the product(s) of the *carORS* region was responsible for the regulation of expression of these biosynthetic genes.

There is also evidence for the control of carotenogenesis beyond the level of transcription, with the pathway subject to repression by carotenoid end products (Martinez-Laborda *et al.*, 1986). This study, however, will concentrate upon the light-inducible control of transcription and in particular on the *carQRS* region.

1.8.3 Genetic analysis of the *carQRS* region:

The expression of the *carQRS* region was examined using an integrative plasmid based promoter probe rather than Tn*5 lac* (Hodgson, in press). This work revealed that the promoter of this region was also light-inducible (Hodgson, 1987). The *carQRS* region was first identified by the Car^C phenotype caused by the insertion of Tn*5*ΩDK1811 (Martinez-Laborda *et al.*, 1986). This phenotype was shown to be caused by the occlusion of the light-inducible promoter of the region, by a constitutive, outward reading promoter within the IS*50L* of this transposon (Hodgson, in press). The occluding promoter was inserted upstream of the *carQRS* region. It constitutively drove expression of the *carQRS* region at five to six times the wild-type level of the light-inducible promoter in the dark.

A model was proposed that envisaged the *carQRS* region encoding two functions (Figure 1.3; Martinez-Laborda and Murillo, 1986; Hodgson, in press). Firstly, under the expression of the *carQRS* region light-inducible promoter (referred to as P^{QRS}), a positive regulator of the genes at *carB* and *carC* was proposed. Secondly, evidence for a negative regulator of this function, encoded by the *carQRS* region and acting at P^{QRS} was also found. The model stated that only the negative regulator was inactivated by light. When the cell was exposed to light, therefore, the negative

regulator was inactivated, thereby allowing full expression of the activator of the biosynthetic genes at carB and carC.

1.6.4 DNA sequence analysis of the carQRS region:

The DNA sequence of the carQRS region was determined to reveal three apparently co-transcribed and translationally coupled genes: carQ, carR and carS (Figure 1.4; McGowan, 1989). A fourth, transcriptionally divergent open reading frame was found upstream of the carQRS region light-inducible promoter and on the other DNA strand.

As described above, the carQRS locus was originally identified by the upstream insertion of a transposon (Tn*5*ΩDK1811). It was shown by sequence analysis that this transposon had, by insertion, disrupted the upstream open-reading frame (at position 865 - see Figure 1.4; McGowan, unpublished). The Car^C phenotype of strain DK1811 was later shown to be caused by a transposon encoded, outward reading promoter (Hodgson, in press). In order to investigate the effect of the disruption alone, therefore, the upstream open-reading frame was interrupted using DNA encoding a kanamycin resistance gene (Gorham H., pers. comm.).

The disrupted DNA was used to replace the wild-type upstream gene on the chromosome. The resulting cells remained viable and no effect upon the carotenogenic phenotype was observed. This open reading frame has, therefore, been named, gufA ("gene of unknown function A"). Although the translational initiation codons of carQ and gufA are only 220 base pairs apart, the gufA gene product does not appear to be involved in carotenogenesis.

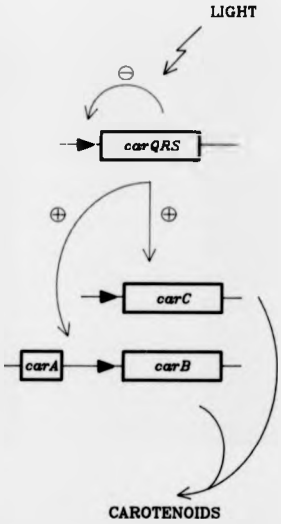
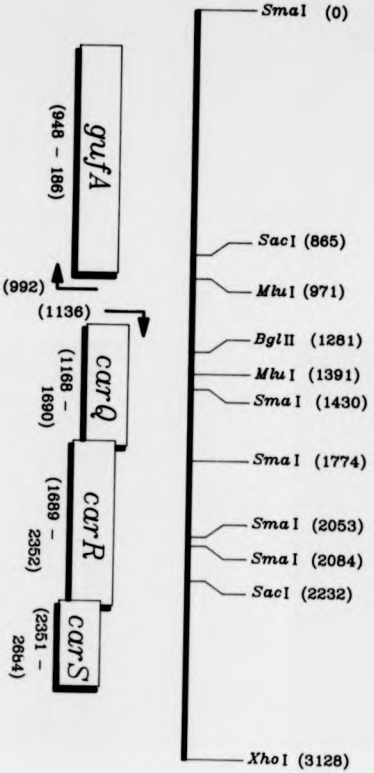


Figure 1.3 Model for the regulation of expression of the carQRS region: (adapted from Hodgson, in press). The carQRS region encodes two functions; an activator of the genes at carB and carC (+), and a negative regulator of the carQRS promoter (-). The negative regulator is inactivated by light. In the presence of light, therefore, activity of the carQRS promoter is no longer prevented and expression of the activator of carB and carC takes place. It is for this reason that the carQRS region promoter has been termed a "light-inducible promoter".

Figure 1.4 Restriction map of the carQRS region of Myxococcus xanthus: Numbers refer to the distance (base pairs) from the end of the sequenced region (McGowan, 1989). The approximate positions of carQ, carR, carS and gufA open reading frames are shown. The positions of the transcriptional start site of both the light-inducible promoter of carQRS (position 1136) and of gufA (position 992) are also indicated (arrows).


SmaI (0)
SacI (865)
MluI (971)
BglII (1281)
MluI (1391)
SmaI (1430)
SmaI (1774)
SmaI (2053)
SmaI (2084)
SacI (2232)
XhoI (3128)
gufA
(948 – 186)
(992)
(1136)
carQ
(1168 – 1690)
carR
(1689 – 2352)
carS
(2351 – 2684)

The transcriptional start site of the light-inducible promoter was determined by primer extension analysis. In the absence of a myxobacterial promoter consensus sequence, comparison of the DNA sequence found immediately upstream of this site was made with consensus promoter sequences of Escherichia coli. This comparison revealed a limited degree of homology between the light-inducible promoter and the E. coli heat shock promoter recognised by the sigma factor, sigma-32 (Figure 1.5; McGowan, 1988). The gufA transcriptional initiation site has also been mapped. Transcription of this gene was found to start only 145 base pairs upstream of the carQRS transcriptional start site.

Figure 1.5 Comparison between DNA sequence found upstream of the light-inducible promoter transcription start site and the sequence recognised by the Escherichia coli heat shock sigma factor, sigma-32: From McGowan, 1988.

1.7 A pathway of events during carotenogenesis: from light reception to biosynthetic enzyme production:

Following the initial proposal of a model for the regulation of the carQRS region and hence carotenogenesis, a number of sequential biochemical and genetic events involved in this regulation have been characterised.

1.7.1 Events occurring before the activation of the carQRS promoter:

Protoporphyrin IX was already thought to be the photosensitiser involved in both photolysis and carotenogenesis (Burchard and Dworkin, 1966; Burchard and Hendricks, 1969). Absorption of a photon of light by ground state protoporphyrin IX leads to the formation of the short-lived singlet state. Intersystem crossover leads to the formation of the more stable, triplet state.

This species of protoporphyrin IX may decay back to the ground state and lose its energy of excitation by the emission of light (fluorescence). Alternatively and as described above, it may lead to the formation of cell damaging singlet oxygen in the same manner as the activated triplet chlorophyll of photosynthetic organisms (Krasnovsky, 1979; Cox and Whitten, 1982; Malik, 1986).

In work carried out for his Ph.D. thesis, Robson has demonstrated the involvement of both photoactivated protoporphyrin IX and one of its reaction products, singlet oxygen, in the activation of the carQRS region light-inducible promoter (Robson, 1982). He confirmed protoporphyrin IX to be present in the M. xanthus wild-type strain, DK101 used in this laboratory. Secondly, the observation of a large increase in expression (1200

fold) of the light-inducible promoter in response to laser light of 410nm (the wavelength of maximum absorption of all porphyrins), further implicated protoporphyrin IX in this process.

Demonstration of the subsequent involvement of singlet oxygen in the activation of the light-inducible promoter was carried out in a number of ways. Firstly, oxygen was shown to be involved with the observation that *in vivo* promoter expression in the dark could be increased approximately two fold by simply increasing the oxygen tension.

Secondly, the addition of hydrogen peroxide or methyl viologen did not result in an increase in the expression of the light-inducible promoter in the dark. This result showed that hydrogen peroxide and the superoxide ion, two other oxygen-reactive species generated by photoactivated porphyrins (Malik *et al.*, 1990), are not involved in the induction of the light-inducible promoter. This was in contrast to the result obtained when toluidine blue is added. This compound, when exposed to red light, produces singlet oxygen (Lang-Feulner and Rau, 1975). When cells in the presence of this compound were exposed to red light, which does not normally cause induction, a large increase in expression of the light-inducible promoter was observed.

Finally, in order to confirm that singlet oxygen was the activating species involved, the effects of adding DABCO (1,4-diazobicyclo [2.2.2] octane), a specific quencher of singlet oxygen (Cox and Whitten, 1982), was examined. DABCO was found to inhibit both the normal light-inducible response of the *carQRS* promoter, as well as the expression in response to toluidine blue under red light.

1.7.2 Conversion of a biochemical stimulus into an activator of carotenogenesis by the carQRS region:

The carQRS region plays a pivotal role in converting a signal derived from photoactivated cellular components, into an activator of transcription of the carotenoid biosynthetic genes. More specifically, singlet oxygen would appear to be able to activate the light-inducible promoter and thereby cause expression of the carQRS region. It presumably achieves this by inactivating the negative regulator of the promoter already proposed (Hodgson, in press), although the precise mechanism of this inactivation remains unclear.

The functions known to be encoded by the carQRS region prior to its sequence analysis were that of: 1) negative regulator of the light-inducible promoter and 2) positive regulator of the biosynthetic genes at carB and carC (Martinez-Laborda and Murillo, 1989; Hodgson, in press). Further work outlined below has implicated the gene products of carR and carS respectively in these roles. No function for the third gene within this region, carQ had yet been ascribed and the determination of a role for its gene product will form a major part of this study.

1.7.3 A negative regulator of the light-inducible promoter is encoded by the carR gene:

DNA sequence analysis of the carQRS regions of three Car^{C} strains has been carried out (McGowan, 1988). In each case, the phenotype was shown to be caused by a single point mutation within the second of the three genes, carR. This seemed to implicate the carR gene product as the

negative regulator of the light-inducible promoter.

The CarR protein however, would appear not to act as a typical repressor. From the hydrophobic domains typical of an integral membrane protein apparent in its derived amino acid sequence, it seems likely that the protein resides within the membrane. Studies in E. coli involving fusions of the CarR protein with protein A of Staphylococcus, have confirmed this inner-membrane association (Gorham, unpublished). In addition, its amino acid sequence does not show any of the motifs normally associated with the ability to bind DNA (for example, the "helix-turn-helix" motif).

It is unlikely, therefore, that the membrane bound CarR protein can act as a typical, DNA binding repressor. Instead, some form of signal transduction between the regulator within the membrane and the light-inducible promoter is envisaged. The nature of this signal, as well as the role of singlet oxygen in the prior inactivation of the CarR regulator, remain unknown.

1.7.4 An activator of carB is encoded by the carS gene:

The 111 amino acid polypeptide encoded by the third of the three genes within the carORS region, carS, has no homology to any known protein. Despite this, there is evidence that carS encodes the already proposed positive regulator of the genes at carB and potentially also carC (in contrast to the model previously published by Martinez-Laborda et al., 1980).

Firstly, a strain was constructed in which expression of carS was driven by the promoter of the kanamycin resistance gene of transposon,

Tn*5* (Hodgson D., pers. comm.). The light-inducible promoter, both *carQ* and *carB*, and the upstream gene, *gufA* had all been deleted. This mutation was shown to be both dominant to the wild-type *carQRS* region and to result in a Car^{c} phenotype. In addition, when transduced into a strain in which the *carB* promoter was fused to a promoterless *lacZ* gene, (strain MB418, Martinez-Laborda *et al*., 1990) this construction was shown to cause constitutive expression of *carB* (Gorham H., unpublished).

Secondly, a strain was constructed in which expression of the *carS* gene was prevented (Hodgson, in press). This was carried out by the integration of a plasmid (pDAH205) into the *M. xanthus* chromosome by homologous recombination at the *carQRS* region. This integration caused the physical uncoupling of expression of *carS* from the light-inducible promoter. The resulting strain was unable to produce carotenoids in response to light-induction.

The confirmation or otherwise of this activation by the *carS* gene product of the genes at *carB* and *carC* is one of the major objectives of this study.

1.7.5 Biosynthetic enzyme production by *carB* and *carC*:

The genes within the *carB* and *carC* loci encode enzymes that are involved early in the pathway of carotenoid biosynthesis (Martinez-Laborda and Murillo, 1988). The *carQRS* activation of genes at *carB* appears to be mediated via the *carA* locus linked to *carB*. This locus has been mapped to a position several kilobases downstream from the *carB* gene (Ruiz-Vazquez *et al*., 1988).

No such linked locus has been described for *carC* and it would appear

that the pattern of expression of this locus differs from that of carB. Expression of carC appears dependent upon growth phase, with maximal expression occurring only during stationary phase (Fontes and Murillo, 1990). Expression of carB is growth phase independent.

Carotenoids that accumulate in strains that have known genetic lesions within carB and carC have been characterised. In this manner, functions for various genes have been ascribed (Martinez-Laborda et al., 1990). Thus, the accumulation of large quantities of phytoene (the first C_{40}, colourless precursor of carotenoids) in a known carC mutant when exposed to light, indicated that the carC gene encodes the enzyme, phytoene dehydrogenase. This enzyme catalyses the stepwise desaturation of the carbon backbone to convert phytoene into lycopene.

Similarly, genes within the carB region have been shown to encode an enzyme(s) involved in the synthesis of phytoene from its precursor, geranylgeranyl pyrophosphate (ggpp). This region has also been shown to encode the gene responsible for the cyclisation of lycopene, carL (Ruiz-Vazquez et al., 1989). Several of the genes encoding key enzymes involved in the biosynthesis of the myxobacton ester have, therefore, been identified.

The carotenoids eventually synthesised by enzymes produced by these loci accumulate within the cell membrane (Mason and Powelson, 1958; Kleinig, 1972). Since this is the site of damage during photolysis (Burchard and Dworkin, 1966), the carotenoids would appear well placed to quench both the triplet photosensitiser and singlet oxygen.

1.8 Project aims:

The major aims of the work described within this thesis were:

(1) to investigate the structure of the light-inducible promoter.

(2) to determine the precise location within the *carQRS* region of mutations that give rise to aberrant carotenoid phenotypes.

(3) to elucidate the function of the first open reading frame, *carQ*.

(4) to investigate the effects of disruption of translational coupling of the two genes, *carQ* and *carR*.

(5) to investigate the nature of the activation of *carB* and *carC* by the *carS* gene product.

Chapter 2.

Methods and Materials.

2.1 Basic Techniques:

2.1.1 Restriction endonuclease digestion of DNA:

DNA in solution was digested with restriction endonuclease enzymes using buffers made according to manufacturers recommendations. Digestion was typically carried out in a volume of between 1µl and 25µl using between 5 and 15 units of enzyme and for around two hours at 37°C. If a partial digestion of DNA was required, a number of reactions were set up each with successive 50% dilutions of enzyme.

2.1.2 Gel electrophoresis of DNA:

Gel electrophoresis was used to visualise and to isolate individual DNA fragments. 0.5% agarose gels were routinely run overnight at a constant 35 Volts. TBE containing 0.5mg/l ethidium bromide was used to buffer the gel. A long-wave UV transilluminator was used to visualise DNA fragments and a Polaroid camera and short-wave UV transilluminator were used to photograph gels. DNA fragments were recovered from gels using the 'Geneclean' kit (Stratech Ltd.). Southern blots of gels and probing of the resulting filters was carried out as described in Maniatis (1982).

2.1.3 Materials needed for gel electrophoresis:

TBE (x10):

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

Loading buffer:

60%	glycerol	0.5%	xylene cyanol
2%	SDS	0.5%	bromophenol blue

2.2 Transformation of Escherichia coli:

2.2.1 Cell competence:

5ml of LB were inoculated with the Escherichia coli strain to be made competent and left with shaking at 37°C overnight. 1ml of this was used to inoculate 100ml of LB in a flask. This was left with vigorous shaking at 37°C for 2½ hours. 50ml of the cells were pelleted by centrifugation in two universal tubes and the supernatant removed. The cells were resuspended in an equal volume of ice cold 0.1M magnesium chloride and pelleted as before. After removal of the supernatant, the cells were resuspended in a total of 25ml ice cold 0.1M calcium chloride. The cells were again pelleted and the supernatant removed. After being resuspended in 2.2ml of ice cold 0.1M calcium chloride, the cells were then left on ice for at least two hours.

2.2.2 Cell transformation:

200µl of competent cells were placed in a 1.5ml microfuge tube and mixed with no more than 50ng of DNA. This was left on ice for 30 minutes before being placed at 42°C for a further 2 minutes. If expression was subsequently required, 1ml of LB was added to the cells and they were placed at 37°C for one hour. The cells were then mixed with 3ml LB soft

agar in a bijou bottle at 55°C and the mixture poured onto an LB agar plate.

2.2.3 Materials needed for transformation:

LB:

10g	tryptone	5g	sodium chloride
5g	yeast extract	Volume to 1 litre with water	
15g	agar (for LB agar)	or 7.5g	agar (for soft agar)

2.3 Preparation of plasmid DNA from Escherichia coli:

2.3.1 Plasmid 'mini-prep' (boiling method):

The mini-prep method of plasmid preparation is based on Holmes and Quigley (1988). An E. coli strain containing the plasmid to be prepared was struck out on an agar plate such that after incubation overnight, the cells formed a patch of approximately 2cm^2. The cells were harvested using a wire loop into a 1.5ml microfuge tube containing 0.33ml STET. After vigorous mixing to resuspend the cells, 0.02ml of the lysozyme mix was added and briefly mixed. The tubes were left on ice for 30 minutes before being placed in a boiling water bath for a further 2 minutes. The tubes were then spun in a bench microfuge for 15 minutes and the cell debris removed with a tooth pick. After making the volume up to 0.33ml with STET, a further 0.33ml of isopropanol was added and mixed. The DNA was then pelleted in a bench microfuge and after removal of the super-

natant, was dried under vacuum. Finally, 0.05ml of water was used to resuspend the DNA.

2.3.2 Materials needed for plasmid mini-prep:

STET:		Lysozyme mix:	
8%	glucose	10mg	lysozyme
0.5%	triton X-100	0.3ml	STET
50mM	EDTA	Prepare same day as use and keep on ice.	
50mM	tris HCl pH. 8.0		

2.3.3 Large scale plasmid prep:

An Escherichia coli strain containing the plasmid to be prepared was used to inoculate 5ml LB and left for approximately 8 hours with shaking at 37°C. 0.5ml of this was then used to inoculate 400ml LB and this was left with shaking at 37°C overnight. The cells were pelleted in a Beckman J2-21 centrifuge for 15 minutes at 10000 rpm (6 x 250ml, JA10 rotor) and the supernatant removed. 40ml ice cold TES was used to resuspend the cells before they were split into two aliquots of 20ml and transferred to polypropylene Oak Ridge tubes. The following refers to one of these aliquots but was carried out on both.

The cells were again pelleted at 10000 rpm for 5 minutes in a MSE "Hi-Spin" 21A centrifuge (8 x 50ml rotor). 10ml of ice cold STE was then used to resuspend the cells after the removal of the supernatant. Cells were routinely stored frozen at -20°C at this stage. One ml freshly

prepared lysozyme solution was then added and mixed by rocking. After 10 minutes on ice, 2.5ml 0.5M EDTA pH. 8.0 was added and mixed by rocking. After a further 10 minutes on ice, 18ml of triton lysis mix was added quickly and mixed by inversion. The mixture was left on ice for 40 minutes before being centrifuged for 35 minutes at 19000 rpm and at 4°C (8 x 50ml rotor). The supernatant was decanted through muslin into a 100ml measuring cylinder containing 28.5g caesium chloride. The caesium chloride was dissolved in the supernatant by swirling and the solution made up to 38ml. After the addition of 2ml ethidium bromide (5mg/ml) the mixture was decanted into a clear Beckman "Quick-Seal" centrifuge tube (25 x 89mm: 39ml). The tubes were topped up with a solution of 71.25% caesium chloride and heat sealed.

The caesium chloride gradient tubes were spun for approximately 17 hours at 45000 rpm and at room temperature using the Beckman L8 Ultracentrifuge (VTi50 rotor). The band of plasmid DNA was visualised using UV light and removed using a 18 gauge needle and syringe.

Ethidium bromide was removed by isopropanol extraction. Five ml of salt-saturated isopropanol was added, the tube inverted several times and the upper isopropanol layer removed. This process was repeated until no colour could be detected in the DNA solution. The volume was made up to 12ml with water, and 24ml ethanol added. After being left at -20°C for several hours, the DNA was pelleted in a centrifuge for 15 minutes at 15000 rpm, the supernatant removed, and the DNA dried under vacuum. The DNA was resuspended in a final volume 0.5ml water.

2.3.4 Materials needed for large scale plasmid prep:

TES:

50mM	tris HCl pH. 8.0
5mM	EDTA
50mM	sodium chloride

STE:

25%	sucrose
50mM	tris HCl pH. 8.0
5mM	EDTA

Triton lysis mix:

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

Lysozyme solution:

10mg/ml	lysozyme
0.25M	tris HCl pH. 8.0

Salt-saturated isopropanol:

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

2.4 Generation of deletions using exonuclease III:

Generation of deletions within DNA plasmids was based upon Henikoff (1984). The commercial 'Erase-a-Base' kit from Promega Ltd. was used according to manufacturers instructions. The treated DNA samples were used to transform E. coli strain MC1061. Plasmid DNA was prepared from the resulting individual colonies using the mini-prep method previously described. The size of the deletion was estimated on a 1% agarose gel following restriction endonuclease digestion and subsequently by DNA sequence analysis.

2.5 DNA sequence analysis:

2.5.1 Single stranded DNA template preparation:

Single stranded DNA was prepared from turbid plaques the day after transformation of *E. coli* strain TG1 with M13 vector DNA. The addition of 0.03ml Xgal (20mg/ml in DMF) and 0.02ml IPTG (24mg/ml) to 3ml of soft agar with the transformed cells, enabled identification of vectors containing inserted DNA. These vectors yielded clear as opposed to blue plaques. Using a tooth pick, cells from a single clear plaque were placed in 1.5ml of LB in test tube. The LB contained a hundredth dilution of an overnight culture of *E. coli* strain TG1. These were incubated for five hours with vigorous shaking at 37°C.

The cells were transferred to 1.5ml microfuge tubes and spun in a bench microfuge for 20 minutes. The supernatant was decanted to a new tube and 0.15ml PEG added. After mixing, the tubes were left at room temperature for 10 minutes. The phage particles were pelleted in a microfuge for 20 minutes and the supernatant removed. 0.1ml water and 0.05ml T.E. saturated phenol were then added to the phage and mixed. After a five minute spin in the microfuge, the upper aqueous layer was transferred into a 0.5ml microfuge tube.

The DNA was precipitated with 0.045ml ammonium acetate and 0.2ml 100% ethanol at -20°C overnight. After pelleting in the microfuge for 15 minutes and removal of the supernatant, the DNA was washed with 0.5ml 100% ethanol and dried under vacuum. Finally, 0.030ml water was used to resuspend the DNA which was stored before use at -20°C.

2.5.2 Materials needed for single stranded template preparation:

T.E.:

10mM tris HCL pH. 8.0 1mM EDTA

PEG:

20g polyethylene glycol (6000 - 7500 mol. wt.)

14.6g sodium chloride

Volume to 100ml with water

The solution was filter sterilised and stored at room temperature.

2.5.3 Preparation of double stranded DNA for sequencing:

In a 0.5ml microfuge tube, 0.018ml double stranded plasmid DNA (prepared from E. coli using the mini-prep method) and 2µl NaOH (2M) were mixed and left at room temperature for five minutes. 8µl ammonium acetate (5M) and 5µl "Glassmilk" (from the commercial 'Geneclean' kit - Stratech Ltd.) were then added to the tube. The contents of the tube were then mixed and left at room temperature for 10 minutes. The tube was then spun in a bench microfuge for 10 seconds and the supernatant removed. The precipitate was resuspended gently in 0.5ml 70% ethanol. The tube was again spun for 10 seconds and the supernatant removed. The precipitate was resuspended in 7µl water, 2µl Sequenase buffer (x10) and 1µl sequencing primer (2ng/µl). The tube was sealed and placed at 55°C for 30 minutes. After this time, the tube was spun for 10 seconds and the supernatant removed to a second 0.5ml microfuge tube. Sequencing of this

template DNA was carried out as below, assuming annealing of the primer to the template DNA had already taken place.

2.5.4 Sequencing reactions:

DNA sequencing was based upon the dideoxy chain termination method described by Sanger et al., (1977). The enzyme "Sequenase" (USB corp., USA) was used according to manufacturers instructions. The fragments produced by this method were separated using a 8% polyacrylamide, buffer gradient gel (the preparation of which has already been described in McGowan, 1988).

After running the gel for around 2½ hours, it was immersed in 10% acetic acid and 10% methanol for 15 minutes. The gel was then transferred to 3MM filter paper, covered with clingfilm, and dried at 80°C under vacuum in a gel drier. The clingfilm was removed and the gel left overnight to expose a sheet of X-ray film.

2.6 Cultivation of Myxococcus xanthus:

2.6.1 Growth conditions:

Myxococcus xanthus strains were cultivated using DCY, either in liquid or on agar plates, at 33°C. Strains on agar plates were stored at 18°C for three weeks before being restruck onto a fresh plate. Strains were stored for longer than this by mixing 0.8ml of a two day old culture with 0.1ml DMSO and freezing at -70°C.

2.6.2 Materials needed for the cultivation of Myxococcus xanthus:

DCY:

20g	casitone (an enzymatic hydrolysate of casein)		
2g	yeast extract	10ml	1M tris HCl pH. 8.0
8ml	1M magnesium sulphate	Volume to 1 litre with water	
15g	agar (for DCY agar)	or 7.5g	agar (for soft agar)

2.7 P1 mediated transduction of Myxococcus xanthus:

2.7.1 P1 lysate generation:

An E. coli P1::Tn9 (P1 cam clr-100; Rosner, 1972) lysogen was incubated with shaking at 30°C overnight in 5ml LB + chloramphenicol (12.5µg/ml). 0.1ml of this culture was used to inoculate another 5ml LB + chloramphenicol (12.5µg/ml) which was incubated with shaking at 30°C until the OD_{660} was 0.21. The culture was then incubated at 42°C with shaking for 35 minutes, followed by 37°C with shaking for two hours. 0.1ml chloroform was added and the culture incubated at 37°C for a further 15 minutes. The lysate was poured into a universal tube and spun at 8500 rpm for 10 minutes. The lysate was decanted into a fresh universal tube and stored at 4°C over chloroform.

To increase both the titre and the stability of the lysate, a further preparatory step was also carried out. In a 1.5ml microfuge tube, 0.2ml of a suitable strain of E. coli was added to 1ml LB + 0.05ml calcium chloride (50mM). 0.1ml of the lysate (diluted to result in just confluent lysis) was then added and the tube incubated at 37°C for 20 minutes. The contents

of the tube were added to 3ml LB soft agar and poured onto a double-T agar plate. The plate was incubated overnight at 37°C. 4mls phage-SM buffer was poured onto the agar plate and the phage were allowed to soak out of the agar overnight at 4°C. The phage suspension was then decanted into a universal tube and stored at 4°C over chloroform.

2.7.2 Materials needed for P1 lysate generation:

double-T agar:		phage-SM buffer:	
20g	tryptone	5.8g	sodium chloride
5g	yeast extract	2g	magnesium sulphate
1ml	(30%) glucose	50ml	1M tris HCl pH. 7.5
10ml	1M calcium chloride	5ml	2% gelatin
1ml	1M magnesium sulphate	volume to one litre with water	
15g	agar		

Volume to 1 litre with water

2.7.3 P1 packaging of plasmid DNA:

An *E. coli* strain containing the plasmid to be packaged was incubated with shaking at 37°C overnight in 5ml LB + kanamycin (40 µg/ml). 0.25ml of this culture was used to inoculate another 5ml LB + kanamycin (40µg/ml) which was incubated with shaking at 37°C until the OD_{660} was 0.21. In a 1.5ml microfuge tube, 5 x 10^8 pfu P1 cam *clr*-100 were added to 0.5ml of the cell culture and left at room temperature for 20 minutes. The contents of the tube were then used to inoculate 5ml LB containing 10mM magnesium chloride, kanamycin (20µg/ml) and chloram-

phenicol (12.5µg/ml). This was incubated at 30°C with shaking overnight.

0.1ml of this culture was used to inoculate 5ml LB containing 10mM magnesium chloride, kanamycin (40µg/ml) and chloramphenicol (12.5µg/ml), and was incubated with shaking at 30°C until the OD_{660} was 0.21. The culture was then incubated at 42°C with shaking for 35 minutes, followed by 37°C with shaking for two hours. 0.1ml chloroform was added and the culture incubated at 37°C for a further 15 minutes. The lysate was poured into a universal bottle and spun at 8500 rpm for 10 minutes. The lysate was decanted into a fresh universal bottle and stored at 4°C over chloroform.

2.7.4 P1 mediated transduction:

0.5ml of a two day old culture of M. xanthus at an OD_{660} of 0.8-1.0 was added to a bijou tube. 0.1ml calcium chloride (50mM), 0.3ml TM and 0.1ml P1 lysate were then added to the tube. The tube was incubated at room temperature for 40 minutes. 2.5ml DCY soft agar was then added to the tube and the mixture poured onto a DCY + kanamycin (20µg/ml) agar plate. The plates were incubated overnight at 33°C. 3ml DCY soft agar + kanamycin (0.8mg/ml) were then used to overlay each plate and the plates incubated at 33°C until colonies became visible (several days).

2.7.5 Materials needed for P1 mediated transduction:

TM:

10mM	tris HCl pH. 8.0	8mM	magnesium sulphate

2.8 Mx4 and Mx8 mediated transduction of Myxococcus xanthus:

2.8.1 Mx phage spot-test:

In order to assay the titre of the Mx phage lysate and to confirm the sensitivity of the donor M. xanthus strain to this phage, a spot-test was carried out. 0.08ml of a two day culture of the donor strain was added to 3ml DCY soft agar at 55°C and poured onto a DCY agar plate. The phage lysate was serially diluted using phage-SM buffer and 0.01ml of each dilution was spotted onto the plate. The plates were incubated at 33°C, after allowing the lysate to first soak into the agar. The extent of growth of the cells at each spot was assessed after two days.

2.8.2 Mx phage transduction:

0.2ml of a two day culture of the donor M. xanthus strain was infected with the Mx phage. Enough phage was added to ensure confluent lysis of the donor strain. The phage were allowed to adsorb to the cells for twenty minutes at room temperature, before the mixture was added to 3ml soft agar at 55°C and poured onto a DCY agar plate. The plate was incubated at 33°C overnight. The phage were "soaked out" of the plate by the addition of 4ml phage-SM and incubation overnight at 4°C. The phage suspension was poured into a bijoux bottle and several drops of chloroform were added. The titre of the resulting phage suspension was assayed by the spot-test procedure described above.

0.2ml of the recipient M. xanthus strain were transduced using around 1×10^8 pfu of this suspension. The phage were allowed to adsorb to the

cells for twenty minutes at room temperature. 0.01ml of the relevant phage antibody was then added to the mixture, before it was plated out as before onto a DCY kanamycin (0.02g/ml) plate. 3ml DCY soft agar + kanamycin (0.8mg/ml) were then used to overlay each plate and the plates incubated at 33°C. Transduced colonies were visible after two further days.

2.8 Preparation of Myxococcus xanthus DNA:

2.8.1 Caesium chloride preparation of DNA:

One ml of a two day old culture of M.xanthus was used to inoculate 100ml DCY. The cells were then incubated for a further two days at 33°C. The cells were pelleted in polypropylene Oak Ridge tubes using a MSE "Hi-Spin" 21A centrifuge (8 x 50ml rotor) at 12000 rpm for 10 minutes. After removal of the supernatant, the cells were resuspended in 10ml STE. 0.05ml proteinase K (20mg/ml) and 2ml 0.5M EDTA were then added and the solutions mixed by inversion. Lysis was brought about by the addition of 1.5ml 10% N-laurylsarcosine and mixing by rapid inversion. The tubes were then placed at 60°C overnight. 28.5g caesium chloride was then dissolved in the mixture before the volume was made up to 38ml with water. Two ml ethidium bromide (5mg/ml) was added and the mixture placed at 4°C for one hour. The mixture was then pelleted at 15000 rpm for 15 minutes (8 x 50ml rotor) and decanted into a clear Beckman "Quick-Seal" centrifuge tube (25 x 89mm - 39ml).

The centrifuge tubes were then treated in the same fashion as the final stages of a large scale preparation of E. coli plasmid DNA - the tubes topped up, sealed and centrifuged in a Beckman L8 Ultracentrifuge

(VTi50 rotor). The chromosomal DNA was removed using a 16 gauge needle and syringe under UV light and ethidium bromide was removed using salt-saturated isopropanol. After precipitation with ethanol, the DNA was centrifuged, dried and resuspended in 1ml water.

2.8.2 Quick preparation of DNA:

5ml of a two day old Myxococcus xanthus culture were pelleted in a universal tube and resuspended in 3ml STE. 0.015ml proteinase K (20mg/ml), 0.6ml EDTA (0.5M) and 0.4ml N-laurylsarcosine (10%) were each added to the cells and mixed by inversion. The universal tube was left standing in a 60°C water-bath overnight. 0.4ml sodium acetate (3M) was added to the contents of the tube and mixed by inversion. 3ml ethanol (100%) was gently added to the mixture and the chromosomal DNA was spooled out using a heat-sealed Pasteur pipette. The DNA was twice washed in ethanol (100%), before being resuspended in 1ml water in an microfuge tube.

It was usually found necessary to further purify the DNA by a second ethanol precipitation. This was carried out by adding 0.05ml sodium acetate (3M) and 1ml ethanol (100%) to half of the DNA suspension in an microfuge tube. The DNA was pelleted in a bench microfuge, washed twice with ethanol (100%), vacuum dried and resuspended in a final volume of 0.5ml water.

2.10 Beta-galactosidase assays of Myxococcus xanthus:

2.10.1 Culture conditions:

2ml of a two day old culture was used to inoculate 250ml DCY (made using purified water, FSA Laboratory Supplies, England) in a round bottle containing a magnetic flea. This was incubated in the dark overnight at 33°C, with vigorous stirring. Polystyrene was used to insulate the culture from the heat generated by the magnetic stirrer. The cultures were incubated overnight before harvesting. If required, light-induction of the culture was carried out by exposure to 125 $\mu E/m^2/s$ using two fluorescent tubes (warm white type) from time zero onwards.

10ml of cells were removed from the culture at each time point. 2ml of this were used to determine the OD_{660}. The remaining 8ml were pelleted in a universal bottle. The supernatant was removed and the cells were resuspended in 8ml *lacZ* buffer. The cells were again pelleted and resuspended in 0.5ml *lacZ* buffer. The cells were frozen in volumes of 10µl, 20µl and 0.5ml in 1.5ml microfuge tubes until the assays were performed.

2.10.2 Protein assay:

The two samples of 0.01ml and 0.02ml were each made up to 0.15ml with water. 0.15ml sodium hydroxide (1M) was added to each tube before they were boiled for five minutes. After allowing the tubes to cool to room temperature, 0.75ml reagent C was added to each tube. The contents of each tube were mixed and allowed to stand for ten minutes. 0.15ml Folin and Ciocalteu's phenol reagent (50%) was then added to each tube

and the contents mixed. After allowing to stand for 30 minutes, the A_{750} was noted. A standard curve was constructed concurrently using BSA at concentrations between 0mg/ml and 150mg/ml.

2.10.3 Beta-galactosidase assay:

This assay was carried out upon cells in the 0.5ml frozen volume. Assays of cells grown in the dark (including light grown cells at time zero), were usually carried out upon samples of 0.1ml (x2) and 0.05ml (x2) and required an assay blank of 0.1ml, all in 1.5ml microfuge tubes. Assays of cells grown in the light were carried out upon samples of 0.05ml (x2) and 0.01ml (x2) and required an assay blank of 0.05ml. All samples were made up to 0.1ml with lacZ buffer and placed on ice. The cells were lysed with the addition of 0.05ml SDS (0.1%) and vortexing. 0.4ml ONPG (1mg/ml in lacZ buffer, freshly made) was added to the contents of the assay tubes. 0.4ml lacZ buffer only was added to the assay blank tubes. All tubes were transferred to a 37°C water bath and the time noted.

When a suitable yellow colour had developed in the assay tubes, they and the assay blanks were removed to room temperature and 0.5ml sodium carbonate (1M) added to the contents. The time taken for the reaction and the A_{420} of the contents of all tubes (using a zero blank of lacZ buffer) were noted. Ideally times would be between 30 minutes and 150 minutes, and the A_{420} would be between 0.6 and 0.8.

The number of arbitrary beta-galactosidase (ß-gal) units in each sample could then be calculated using the equation given below. A program written in BASIC for use on the Amstrad PCW computer was routinely used for calculation of ß-gal units (appendix 1). The number of units were

derived for each of the samples at each time point and graphs have been plotted using the mean of these values. Error bars are only shown on graphs where the mean differed from any of the values by more than 10%.

beta-gal units/sample =

$$\frac{A_{420}\text{ corrected} \times \text{vol. (µl) used in protein assay} \times 233.333}{\text{protein (µg)} \times \text{vol. (µl) used in } \underline{lacZ} \text{ assay} \times \text{time (min.) } \underline{lacZ} \text{ assay}}$$

(where A_{420} corrected = A_{420} assay - A_{420} assay blank)

2.10.4 Materials needed for beta-galactosidase assay:

lacZ buffer:

8.52g	di-sodium hydrogen phosphate (anhydrous)
8.24g	sodium di-hydrogen phosphate (.2 water)
0.245g	magnesium sulphate (.7 water)
0.75g	potassium chloride Volume to 1 litre with water

2.11 Amplification of M. xanthus DNA using the polymerase chain reaction:

Chromosomal DNA isolated from Myxococcus xanthus using the quick method of preparation (Section 2.8.2) was diluted 1:100 with water. 5µl of the diluted DNA was added to a 0.5ml siliconised microfuge tube containing the following: 5µl Taq buffer (supplied by manufacturers), 5µl NTP's (dATP, dCTP, dGTP and dTTP, each at 1mM), 3µl magnesium chloride (25mM), 2.5 µl each primer (each at 20ng/µl), 27µl water.

0.05ml paraffin oil was added to the contents of the tube, which was

then placed at 94°C for five minutes. The temperature of the tube was then lowered to 85°C and 0.1µl *Taq* polymerase added. The temperature of the tube was then lowered again, this time to the annealing temperature (for example, using *M. virescens* DNA and the primers CarR-2 and ORFX-2, 52°C) for two minutes. The temperature of the tube was then raised to 72°C, again for two minutes. Thirty cycles of 94°C for 1 minute, annealing temp for two minutes and 72°C for two minutes were then used to amplify the DNA.

2.12 Plasmids constructed:

The plasmids constructed during the course of this study are listed below. The plasmid name is followed by a brief description of the important features of the plasmid and by details of its construction.

pSJM100 - p^{QRS}, truncated *carQ*, Ap^r.
- pIC19R (Marsh *et al.*, 1984) *SacI*/*EcoRV* = 2688bp + pDAH238 (Hodgson D., pers. comm.) *SacI*/*SmaI* = 565bp.

pSJM101 - promoterless *lacZ*, P1 *inc*, Mx8 *attP*, Km^r.
- pDAH274 (Scanlan *et al.*, 1986) *PstI* partial digestion = 12270bp + pPR107 (Robson, 1982) *PstI* partial digestion = 5700bp.

pSJM103 - promoterless *lacZ*, P1 *inc*, Mx8 *attP*, Km^r.
- pSJM101 *KpnI* + religation.

pSJM105 - p^{QRS} driving promoterless *lacZ*, P1 *inc*, Mx8 *attP*, Km^r.
- pSJM103 *EcoRI* = 18000bp + pSJM100 *EcoRI* = 815bp.

pSJM106 - as pSJM105 but insert in opposite orientation. *sufA* promoter therefore drives expression of promoterless *lacZ*.

pSJM107 - P^{QRS} drives carQ & promoterless lacZ, P1 inc, Mx8 attP, Km^r.
- pSJM103 EcoRI = 18000bp + pSJM3 (McGowan, 1988) EcoRI = 884bp.

pSJM108 - P^{QRS}, Ap^r.
- pMTL24 (Chambers et al., 1988) MluI = 2518bp + pSJM105 MluI = 420bp.

pSJM109 - P^{QRS} driving promoterless lacZ, P1 inc, Mx8 attP, Km^r.
- pSJM103 EcoRI = 18000bp + pSJM108 EcoRI = 548bp.

pSJM110 - Ap^r, P1 inc, carQRS(carQ1) region derived from strain MR132 (Martinez-Laborda & Murillo, 1988).
- self-ligation of SalI digested chromosomal DNA.

pSJM111 - as pSJM110 but derived from strain MR151 (Balsalobre et al. 1987), therefore carR3.

pSJM112 - as pSJM110 but derived from strain MR171 (Martinez-Laborda & Murillo, 1988), therefore carQ2.

pSJM113 - as pSJM110 but derived from strain MR448 (Murillo F., pers. comm.), therefore carR7.

pSJM114 - as pSJM110 but derived from strain MR448 (Murillo F., pers. comm.), therefore carR8.

pSJM115 - as pSJM110 but derived from strain MR486 (Murillo F., pers. comm.), therefore carS1.

pSJM116 - as pSJM107 but insert in opposite orientation. sufA promoter therefore drives expression of promoterless lacZ gene.

pSJM122 - P^{QRS}, carQ(carQ2), truncated carR, car, 1850, Km^r, Ap^r, P1 inc.
- pDAH218 (Hodgson D. pers. comm.) SacI = 13210bp + pSJM112 SacI = 1355bp.

pSJM127 - Km^r, 3' end of carB, carS(carS1). Ap^r, P1 inc.

- pDAH142 (Hodgson, in press) SmaI = 13850bp + pSJM115 SacI/XhoI (filled) = 802bp.

pSJM130 - 3' end of carQ, carRS. Ap^r.

- pMTL21 (Chambers et al, 1988) XhoI/MluI = 2503bp + pDAH193 (Hodgson, in press) XhoI/MluI = 1743bp.

pSJM132 - 3' end of carQ, carR, Ap^r.

- pSJM130 AvaII partial digestion and religation.

pSJM133 - 3' end of carQ, carR, Ap^r.

- pSJM132 XhoI = 3956bp + pDAH201 (Hodgson D., pers. comm.) XhoI = 2500bp.

pSJM134 - P^{QRS}, carQ(carQ2), truncated carR. Ap^r.

- pIC19R SacI = 2695bp + pSJM112 SacI = 1355bp.

pSJM136 - P^{QRS} driving expression of carQ(carQ2), truncated carR & promoterless lacZ, P1 inc, Mx8 attP, Km^r.

- pSJM103 EcoRI = 18000bp + pSJM134 EcoRI = 1414bp.

pSJM138 - P^{QRS}, Ap^r.

- pIC19R ClaI = 2695bp + pSJM100 TaqI = 249bp.

pSJM139 - P^{QRS} driving expression of promoterless lacZ, P1 inc, Mx8 attP, Km^r.

- pSJM103 EcoRI = 18000bp + pSJM138 EcoRI = 312bp.

pSJM140 - 3' end of carQ, carR, Ap^r, Km^r, P1 inc.

- pDAH180 (Hodgson D., in press) HindIII/EcoRI = 11470bp + pSJM133 HindIII/EcoRI = 4020bp.

pSJM145 - as pSJM134 but derived from pSJM110, therefore carQ(carQ1).

pSJM146 - as pSJM136 but derived from pSJM145, therefore carQ(carQ1).

pSJM147 - carC promoter driving expression of promoterless lacZ. F1 inc. P^{QRS} driving expression of carQ. Km^r.

- pMAB206 (Fontes M., pers. comm.) ScaI = 14870bp + pSJM3 EcoRV/PvuII = 953bp.

pSJM149 - F1 inc, carQRS(carS1) region, Mx8 attc. Km^r.

- pSJM115 ScaI/XhoI (filled) = 10230bp + pSJM103 BamHI (filled) = 8730bp.

2.13 Escherichia coli strains used:

ED8812 - thr, leu, thi, lacZ(M15), lacY $\bar{}$, λ^s, tonA, supE, hsdR$^-$, hsdM$^-$

GM48 - dam, dcm, thr, leu, ara, tonA31, lacY, tsx-78, gln, gal, thi

MC1061 - hsdR, mcrB, araD139, Δ(ara-ABC-leu)7679, galU, galK, rpsL, thi, lacX74(lacIPOZY)

TG1 - supE, hsd D5, thi, Δ(lac-proAB)
F'[traD36, proAB$^+$, lacI^q, lacZ(M15)]

2.14 Myxococcus xanthus strains used:

Myxococcus xanthus strains constructed or used during the course of this study are listed below. The strain name is followed by the phenotype, genotype, a brief description of strain construction (when appropriate), and the reference.

MR132 - Car$^-$. carQ1, carR4 (Martinez-Laborda and Murillo, 1989).

MR135 - Car$^-$. car12 (A large deletion of the chromosome has removed the whole of the carQRS region). (Martinez-Laborda and Murillo, 1989).

MR148 - Car$^-$, Tc^r. carB1 (Tn*5-132 insertion into carB region). (Martinez-Laborda et al., 1986).

MR151 - Car^C. carR3 (Balsalobre et al., 1987)

MR171 - Car$^-$. carQ2, carR4 (Martinez-Laborda and Murillo, 1989).

MR401 - Car$^-$, Km^r. carB2 (Tn*5 lac insertion into the carB region. One of the promoters within the carB region drives expression of a promoterless lacZ gene). (Balsalobre et al., 1987).

MR418 - Car$^-$, Tc^r. carB2 (in situ replacement of the Km^r gene of Tn*5 lac of MR401 by the Tc^r gene of Tn*5-132. One of the promoters within the carB region drives expression of a promoterless lacZ gene). (Martinez-Laborda et al., 1986).

MR446 - Car^C. carR7 (Murillo F., pers. comm.)

MR448 - Car^C. carR8 (Murillo F., pers. comm.)

MR486 - Car$^+$. carS1 (Murillo F., pers. comm.)

UWM300 - Car^-, Km^r. plasmid pSJM140 integrated into the chromosome of the wild-type strain, DK101 by homologous recombination at carQRS region. The second copy of the carQRS operon encoded by this plasmid carries the carS2 mutation. (This study).

UWM301 - Car^-. carS2 (This study).

UWM303 - Car^-. carQ2 (This study).

DK101 - Car^+ (laboratory wild-type strain of M. xanthus). (Hagen et al., 1978).

2.15 Other myxobacterial species used:

The other species of myxobacteria used during the course of this study are listed with their strain names and source.

Myxococcus virescens, strain Mxv4 (Reichenbach H., pers. comm.)
Myxococcus fulvus, strain Mxf2 (Reichenbach H., pers. comm.)
Myxococcus stipitatus, strain Mxs2 (Reichenbach H., pers. comm.)
Stigmatella aurantiaca, strain DW4 (Hartree D., pers. comm.)
Stigmatella aurantiaca, strain Sga1 (Reichenbach H., pers. comm.)
Cystobacter fuscus, strain Cbf16 (Reichenbach H., pers. comm.)

RESULTS

Chapter 3.

Structure of the Light-Inducible Promoter of carQRS.

3.1 Introduction:

The transcriptionally divergent open reading frames of carQ and gufA are only 220bp apart. Primer extension studies of mRNA has further increased our knowledge of the promoters of these genes (McGowan, 1988). Transcription of gufA is thought to start at position 991, (see Figure 1.4) whereas transcription of carQ is believed to start 145bp away at position 1136. The object of work presented in this chapter was the characterisation of the light-inducible promoter region.

This analysis was carried out using two different approaches. We attempted to use the polymerase chain reaction to amplify DNA encoding the carQRS regions of other species of Myxococcus. The work described first, however, involved determination of the minimum sequence within the region that encoded the light-inducible promoter. This was attempted using a nested series of deletions across the promoter region, created in vitro. Members of this deletion series were then assayed in vivo for retention of their ability to cause promoter activity in response to light.

In order to assay promoter activity, we modified an existing promoter probe, pDAH274 (Scanlan et al., 1990). This plasmid already encoded a promoterless lacZ gene, upstream of which were several unique restriction enzyme sites. The promoter activity of a fragment cloned into one of these sites could be assayed, therefore, by the amount of β-galactosidase produced by the lacZ gene.

The plasmid, in addition, encoded the incompatibility region of the coliphage, P1 (P1 inc). P1, whilst able to inject its DNA into M. xanthus, can neither replicate, nor enter lysogeny. The P1 inc fragment ensures the efficient packaging during lytic growth, of a co-integrate formed between

P1 and the plasmid, by recombination at their homologous P1 inc regions. The efficient transduction of M. xanthus using the P1 packaged probe is thereby ensured (Shimkets et al., 1983). Cells that have been transduced by the plasmid, may be selected for using the plasmid encoded kanamycin resistance determinant.

The plasmid was modified by the insertion of DNA encoding the site-specific recombination system of the bacteriophage Mx8 (attP: Orndorff et al., 1983). The insertion of this fragment allows integration of the plasmid into the M. xanthus chromosome by site-specific recombination at the Mx8 attachment site, attB. Integration of plasmids normally relies upon homology between the incoming DNA and the chromosome. Because this would be very inefficient for small fragments (less than 500bp), this modification greatly improves the efficiency of the integration step.

Use of this plasmid has other advantages over the normal integration of fragments. It does not destroy the recipient carQRS genes by insertional inactivation and, therefore, allows the modified promoter region to be assayed in an essentially wild-type background. It also enables the transfer of the fragments into strains of M. xanthus in which the carQRS operon has been deleted. In addition, gene conversion only occurs at a greatly reduced frequency following site-specific recombination (Li and Shimkets, 1988). Although this was not a problem in these experiments, gene conversion was significant using a homology based, integrative plasmid (later chapters).

3.2 Deletion analysis of the light-inducible promoter:

3.2.1 Construction of plasmid pSJM103:

The modification of the promoter probe, pDAH274, was carried out in two stages (Figure 3.1). The attP fragment of Mx8 encoding the site-specific recombination system had previously been cloned in a plasmid named pPR107 (Robson, 1992). This fragment, of approximately 5.6Kb, was isolated using a partial PstI digest. It was sub-cloned into the promoter probe pDAH274, also partially digested using PstI and the resulting plasmid named pSJM101. In the second stage, pSJM101 was digested with KpnI and religated in order to leave an unique EcoRI site in front of the promoter-less lacZ gene. It was into this unique site that all promoter fragments to be tested were subsequently cloned.

The plasmid, pSJM103 with no inserted fragment, was used to transduce the wild-type strain of M. xanthus, DK101 using P1. The background level of β-galactosidase at attB produced by the resulting strain was assayed in both the light and the dark as a control for the experiments described below (Figure 3.2).

3.2.2 Construction of a deletion series within the carQRS promoter:

The deletion series within the carQRS region was constructed using the enzyme exonuclease III. Deletions were made in a 565bp fragment that had been ligated into the plasmid, pIC19R. This fragment was isolated from pDAH238 by digestion with the restriction enzymes SacI at position 865 and SmaI at position 1430 (see Figure 1.4).

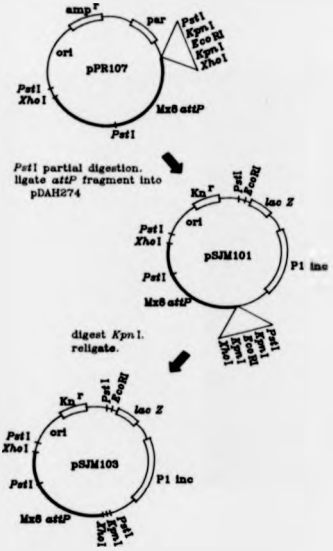


Figure 3.1 Modification of the promoter probe, pDAH274: A fragment of approximately 5.6Kb encoding the site-specific recombination system of Mx8 (attP) was isolated following a partial PstI digest of plasmid, pPR107. This fragment was sub-cloned into the promoter probe, pDAH274, also partially digested using PstI, and the resulting plasmid was named pSJM101. This plasmid was then digested with KpnI and religated in order to leave an unique EcoRI site in front of the promoterless lacZ gene. It was into this site that all fragments to be tested were subsequently cloned.

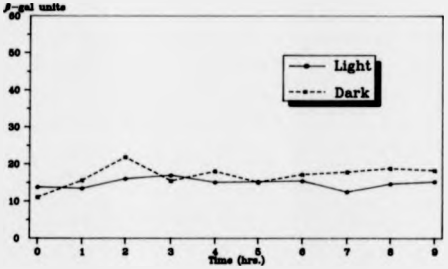


Figure 3.2 β-galactosidase activity of strain DK101::pSJM103: The promoter probe, pSJM103, contains no insert and the activity measured, therefore, is due to the background promoter activity at the Mx8 attB site.

The fragment encoded the promoter of the carQRS operon (P^{QRS}) and part of the first gene, carQ. The resulting plasmid was named pSJM100.

Other deletions were made in a 420bp fragment. This fragment was isolated from pSJM100 by digestion with the enzyme MluI at positions 971 and 1391. It was ligated into plasmid pMTL24 (Chambers et al., 1988) digested with the same enzyme and the resulting plasmid was named pSJM108.

Exonuclease III digests one strand of double stranded DNA at a 5' overhanging end by processively removing nucleotides from the 3' end. It does not digest a 3' overhanging end. In the construction of both pSJM100 and pSJM108, the fragments were inserted such that digestion with exonuclease III, following digestion with two suitable restriction enzymes, would lead to the deletion of P^{QRS} from the distal end. The plasmid, pSJM100 was digested, therefore, with HindIII and PstI prior to treatment with exonuclease III. Only the recessed 3' end generated by HindIII was attacked by exonuclease III and, thus, only promoter DNA and not vector DNA was digested.

Similarly, pSJM108 was digested with XhoI and SohI to allow exonuclease III digestion at the recessed 3' end generated by XhoI. It has been reported that the 3' end generated by SohI offers better protection against attack by exonuclease III than that generated by PstI (Promega Ltd., Notes, Number 24).

Following digestion with exonuclease III, The large overhanging 5' end was removed using S1 nuclease, the ends of the plasmids were filled using the Klenow fragment of DNA polymerase I and the plasmids were self-ligated. The plasmids were then used to transform the E. coli strain MC1061. By varying the time allowed for exonuclease III digestion before

ligation of each of the plasmids, we were able to create different sized deletions within the fragments. Times of up to one minute were used, with most deletions of the required size being generated after only 20 seconds digestion using exonuclease III.

The extent of the deletion created was assayed in each case by DNA sequence analysis of the fragments after the re-isolation of plasmid DNA from transformed colonies. Fragments from pSJM100 derived plasmids were isolated by digestion using EcoRI, cloned into M13 and sequenced. Fragments from pSJM108 derived plasmids were directly plasmid sequenced using the M13 "-17", universal primer.

Using the above described method, a deletion series across the light-inducible promoter was created. Some of these deletions end-points were very close to others (within 1 to 4bp) and, therefore, were not used in subsequent analysis of the promoter activity.

3.2.3 light-inducible promoter activity of deleted fragments:

The promoter activities of the undeleted fragments of both pSJM100 and pSJM108 (with end-points 271bp and 165bp upstream of the carO transcription start site respectively) were assayed in vivo using the modified promoter probe, pSJM103. The two fragments, isolated following digestion with EcoRI, were ligated into this probe at the unique EcoRI site. The resulting plasmids were named, pSJM105 and pSJM109 respectively. Following P1 mediated transduction of the wild-type strain of M. xanthus, DK101, the levels of β-galactosidase produced by the two resulting strains were assayed in both the dark and the light (Figures 3.3 and 3.4).

In addition, the promoter activity of a third fragment was assayed

using the promoter probe. The 248bp TaqI/TaqI fragment of pSJM100 (from position 953 to position 1202) was cloned into plasmid, pIC19R digested with ClaI. The end-point of this fragment was 183bp upstream of the transcription start point and the resulting plasmid was named pSJM138. The fragment was subsequently re-isolated using EcoRI and cloned into pSJM103. Again, the level of β-galactosidase was assayed after the resulting plasmid, pSJM139, had been used to transduce strain DK101 using P1 (Figure 3.5).

All three fragments were shown to be able to promote expression of the lacZ gene following exposure of the cells to light. This activation, however, was far below that of the same promoter when assayed at the carQRS region (Figure 3.6). In addition, the level of activity when assayed in the dark would appear to be between two and three times the wild-type uninduced activity.

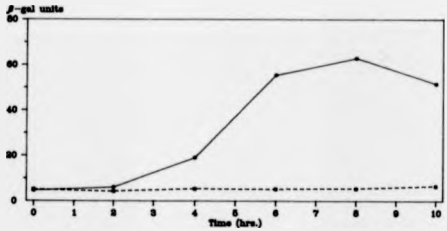
Nine of the deleted promoter fragments were also assayed in vivo for expression of lacZ using the modified promoter probe, pSJM103 (data not shown), and were found to fall into two classes. The results are summarised in Figure 3.7. Some were shown to be able to promote expression of lacZ in response to light, whereas others had lost this ability.

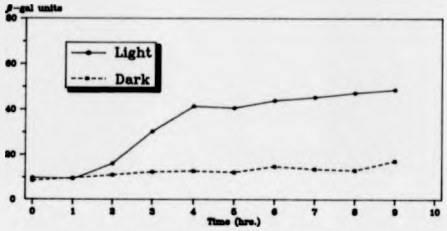
The two fragments whose end-points were 145bp and 156bp upstream of the carQ transcription start ("-145" and "-156") exhibited similar levels of expression of lacZ to the undeleted fragments of pSJM100 (at -185bp; data not shown). When more DNA was deleted, no light-induced expression was observed. Thus, the fragment whose end-point was at -136bp and all those nearer to the transcription start site, could not promote expression of the lacZ gene in response to light.

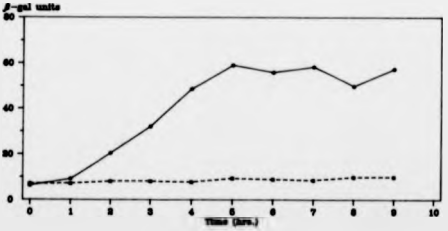
Figure 3.3 β-galactosidase activity of strain DK101::pSJM165: The light-inducible promoter activity of a fragment integrated at attB and whose end-point was 271bp upstream of the carQ transcription start point was assayed.

Figure 3.4 β-galactosidase activity of strain DK101::pSJM109: The light-inducible promoter activity of a fragment integrated at attB and whose end-point was 185bp upstream of the carQ transcription start point was assayed.

Figure 3.5 β-galactosidase activity of strain DK101::pSJM139: The light-inducible promoter activity of a fragment integrated at attB and whose end-point was 183bp upstream of the carQ transcription start point was assayed.


β-gal units
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Time (hrs.)


β-gal units
80
60
40
20
0
Light
Dark
0 1 2 3 4 5 6 7 8 9 10
Time (hrs.)


β-gal units
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Time (hrs.)

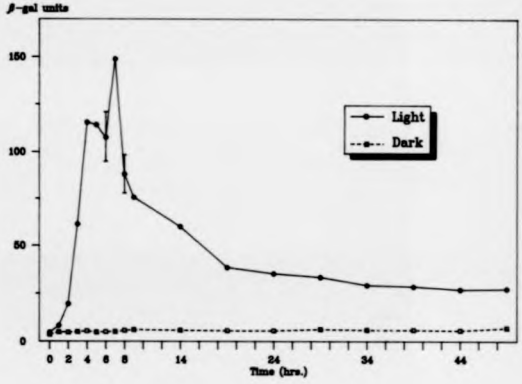
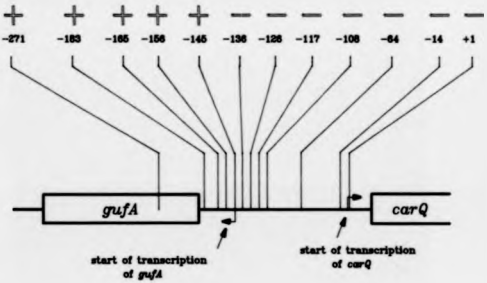


Figure 3.6 β-galactosidase activity of strain DK101::pDAH217: The wild-type light-inducible promoter activity of the carQRS promoter was assayed.

Figure 3.7 Deletion end-points of fragments assayed for light-inducible promoter activity: Deletions were generated within the light-inducible promoter region by digestion of DNA processively from the 5' end using exonuclease III. The figures above each end-point refer to the distance (bp's) upstream from the transcription start of carQ. The notation above each figure (in red) indicates whether the fragment was able to promote transcription in response to light (+), or had lost this ability by virtue of the deletion (-).



3.2.4 *gufA* promoter expression:

In the construction of plasmid, pSJM105 (Section 3.2.2), it was possible for the P^{QRS} encoding fragment to be inserted in the opposite orientation in pSJM103. Because this fragment also encoded the promoter of transcription of *gufA* on the opposite strand, in this orientation the *gufA* promoter caused expression of the promoterless *lacZ* gene at the Mx8 attachment site.

A plasmid resulting from such an event was isolated and named pSJM106. Following P1 mediated transduction of strain DK101 with this plasmid, β-galactosidase assays were carried out in the light and the dark (Figure 3.8).

3.2.5 Analysis of results:

The minimum region required for light-inducible expression of *carO*, upstream of the transcription start site, is between 138bp and 145bp. Because such a large region of DNA would appear to be involved in the light-inducible expression, only limited conclusions may be drawn.

Light-inducible expression from P^{QRS} would not appear, for example, to rely solely upon RNA polymerase. This possibility had been considered upon analysis of the DNA sequence of the *carQRS* region. It had been noted that the sequence upstream of the *carO* transcription start site was 58% homologous to the heat shock promoter consensus sequence recognised by the sigma-32 factor of *E. coli* (McGowan, 1989; Figure 1.5). Had expression relied upon a particular form of RNA polymerase alone, we might have expected a very much smaller region, perhaps only to -45bp, to

be the minimum required for activity. This does not preclude a role for a specific sigma factor in expression from this promoter. If involved, however, it is likely to be acting in concert with another, as yet unknown factor.

As described in Section 3.1, 145bp upstream of the carQ transcription start is precisely the point from which transcription of gufA is believed to initiate. From the data described, therefore, expression of the light-inducible promoter appears to require the presence of the whole of the promoter of the upstream gene, gufA.

One possible conclusion from this observation is that the transcription of gufA is required before transcription of carQRS is possible (as described in section 1.6.4, the gufA gene product itself would appear to be uninvolved in this process). In this regard, it is interesting to note that the promoter of gufA exhibits constitutive expression and does not alter this expression significantly upon illumination (Figure 3.8). Inactivation of the gufA promoter by mutagenesis to determine whether there is concurrent loss of activity by the carQRS promoter, is one obvious future area of study arising from these observations.

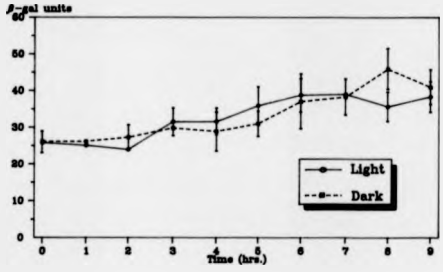


Figure 3.8 β-galactosidase activity of strain DK101::pSJM106: The plasmid, pSJM106 integrated into the chromosome at the Mx8 attachment site (attB) of the wild-type strain, DK101 and encodes the promoter of gufA driving expression of lacZ. (Amount of protein was assayed using the "BIORAD" protein assay kit, Bio-Rad Ltd., rather than that described in Section 2.10.2).

3.3 The light-inducible promoters of other species of myxobacteria:

3.3.1 PCR amplification of chromosomal DNA:

In addition to M. xanthus, other species of myxobacteria have been shown to produce carotenoids in response to light (Reichenbach & Kleinig, 1971). We assumed that these species would regulate this response via the products of genes analogous to those of the carQRS operon of M. xanthus. We attempted, therefore, to use the polymerase chain reaction (PCR; Saiki et al., 1985, 1988) to amplify DNA encoding the carQRS regions of some of these species.

Analysis of P^{QRS} using the polymerase chain reaction was initially attempted using DNA derived from Myxococcus virescens. This species is most closely related to M. xanthus (Reichenbach and Dworkin, 1981; Reichenbach, 1984) and is, therefore, most likely to contain regions of greatest similarity.

Chromosomal DNA from M. virescens strain Mxv4 (Reichenbach H., pers. comm.) was isolated using the spooling method described in Chapter 2. Part of the carQRS region encoded by this DNA was then amplified using the enzyme Taq polymerase and the primers, CarR-2 and ORFX-2 (McGowan, 1988; Table 3.1). These primers are homologous to the coding regions of carQ and gufA respectively.

Following isolation by gel electrophoresis, the PCR products of two separate PCR reactions were each end-filled using the Klenow fragment of DNA polymerase I and T4 DNA polymerase. They were then ligated into M13 mp10 digested with SmaI. These were then used to transform E. coli, strain TG1 and the resulting single stranded DNA was sequenced.

3.3.2 DNA sequence analysis of promoter region:

The DNA sequence of the M. virescens promoter region was compared to that of M. xanthus (Figure 3.9). Over the 403bp region compared, 25 differences between the two sequences were found. These changes were split evenly between transitions and transversions, with 12 examples of the former event found and thirteen of the latter. No additions or deletions were found.

Table 3.1 Primers used in the PCR amplification of myxobacterial DNA:

primer	position (5'-3')	sequence (5'-3')
CarR-1	1218 - 1199	CGCGAAGAGGTCCTCGAAGG
CarR-2	1258 - 1239	CCATCCGGGCCAGGAAGCCT
ORFX-1	896 - 915	CCCAGTCCCGTCGCCGTGCC
ORFX-2	856 - 875	CTTCCGGCTGAGCTCCGAGG

In order to overcome problems involving misincorporation of nucleotides during amplification by taq polymerase, DNA sequence analysis of two independent PCR reaction products was carried out. A comparison between these two sequences revealed only a single base difference at position 1023. Without further DNA sequence analysis, it is not possible to

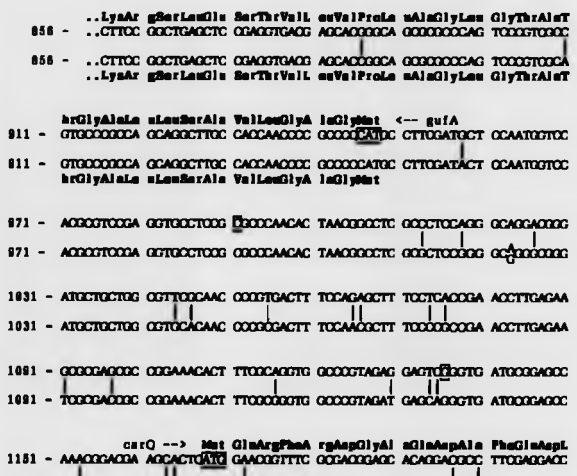
determine whether the base in *M. virescens* in this position, is adenine (the same as *M. xanthus*) or guanine. In addition, the DNA sequence obtained from one of the two independently amplified reaction products was not complete. We were, therefore, unable to confirm one of the 25 base changes between *M. xanthus* and *M. virescens* at position 886 within the *gufA* open reading frame.

In summary, with the exception of the bases identified at positions 886 and 1023, we are confident that we have identified at least 24 differences between part of the *carQRS* region of *M. xanthus* and *M. virescens*, and that these are not artifacts introduced during amplification. These changes must result from mutational events that have occurred since the two species diverged during their evolution.

Although almost half of the sequence is made up of part of the coding regions of *carQ* and *gufA*, only four of the 24 differences were found in these areas. Of these four, only one led to a change in the coded amino acid. The second codon of *carQ* in *M. xanthus* encodes glutamate, whereas the codon in this position in *M. virescens* is for valine. It would appear, therefore, that since the two species diverged, there has been greater selection against mutations occurring within the two open reading frames, than within the intervening DNA. This would not be unexpected, because mutations that led to an amino acid change may be deleterious to the correct functioning of the two proteins.

The corollary to the above argument is that the higher proportion of mutations in the DNA between the two open reading frames, reflects their relative redundancy. We may suppose, therefore, that the mutations that have occurred within this region are to bases that are largely uninvolved with the functioning of either P^{QRS}, or the promoter of *gufA*.

Figure 3.9 Comparison between the light-inducible promoter regions of M. xanthus and M. virescens: Differences between the DNA sequences of M. xanthus (upper sequence) and that of M. virescens (lower sequence) have been marked ('|'). Numbers refer to the position of the sequence within the M. xanthus carQRS region (McGowan, 1988; see Figure 1.4). The start of transcription of sufA and carQ (891 and 1136 respectively) and their translation start points (positions 948 and 1168 respectively) have each been boxed.



3.3.3 Homology to the heat shock promoter of *Escherichia coli*:

The previously identified homology with the heat shock promoter of *E. coli*, upstream of the *carO* transcription start site of *M. xanthus* (between positions 1098 - 1105 and 1121 - 1129) is also found in *M. virescens* (Figure 3.10). The functional significance of this homology, however, remains far from clear. The results of a separate experiment are interesting to note in this regard.

A sigma factor that has high homology to the heat shock factor of *E. coli* has been found in both *M. xanthus* (Apelian and Inouye, 1990) and *Stigmatella aurantiaca* (Skladny and Schairer, 1992). A strain of *M. xanthus* in which the gene encoding this sigma factor (*sigB*) had been deleted, recently became available (Inouye S., pers. comm.). Although no information was available as to the nature of the response of this strain to heat shock, we were interested to see whether this strain could still induce carotenogenesis in response to light.

Upon exposure to light, both the *sigB* deleted strain and the parental strain, DZF1, were able to mount a wild-type carotenogenic response (data not shown). This particular sigma factor would appear to be uninvolved, therefore, in expression of *carQRS* by the light-inducible promoter. Because we do not know the function of this particular sigma factor, or how many other sigma factors are encoded by myxobacteria, it is still not possible to ignore this homology.

3.3.4 Other myxobacteria:

Chromosomal DNA from a number of different species of myxobacteria (M. xanthus, M. virescens, M. fulvus, M. stipitatus, Cystobacter fuscus and Stigmatella aurantiaca) was digested individually with PstI and SalI and run on an agarose gel, before being blotted onto nitrocellulose. The resulting blot was probed with a fragment of the carQRS region derived from M. xanthus (Figure 3.11).

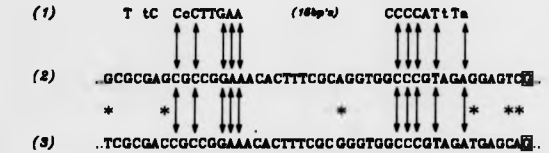


Figure 3.10 Possible RNA polymerase binding sites of the carQRS operons of M. xanthus and M. virescens: The 45 base-pairs immediately upstream of the carQ transcription start site (boxed) of M. xanthus (2) and M. virescens (3), as well as the sequence recognised by the E. coli heat shock sigma factor, sigma-32 (1), are shown. Despite the differences in sequence between these species (shown as '*'), the homology with the heat shock promoter ('↕') remains.

The results of this experiment appeared to show that, with the exception of M. virescens, none of the other species of myxobacteria have regions of close homology with the carQRS region of M. xanthus.

Despite this result, the amplification of DNA encoding the carQRS regions of these myxobacteria was also attempted. All four combinations of the primers CarR-1 or CarR-2 and ORFX-1 or ORFX-2 were used in this experiment. We were unable, however, to amplify DNA that was homologous to the carQRS region of M. xanthus (as confirmed by blotting and probing of PCR reaction products) from any of the chromosomal DNA preparations (data not shown).

3.4 Discussion:

3.4.1 A PCR based experimental approach:

This method has enabled us to identify 16 (possibly 17) differences between the light-inducible promoters of M. xanthus and M. virescens (assuming that, like M. xanthus, the light-inducible promoter of M. virescens initiates transcription at the same position and is encoded by the 145 base-pairs immediately upstream of this site). This number of differences, however, does not enable us to identify the specific base-pairs involved in light-inducible promoter activity. Such regions of homology between the promoters would only have become apparent with a larger number of differences between the two species, or with the comparison of regions from a number of organisms.

Despite the success in amplifying the carQRS region of Myxococcus virescens, an approach based upon PCR suffers from a number of draw-

backs. Obviously, without an analysis of the genes involved, we do not know if the primers chosen are homologous to regions of high conservation. Such regions are more likely to be found in the chromosomes of more distant relatives and are, therefore, of great value when designing PCR primers.

In addition, the primers chosen for the experiments involving PCR were homologous to the open reading frames of carO and the upstream gene, gufA. We have already reported evidence that suggests that GufA is not involved in the carotenogenic response (Section 1.8.4). It is possible, therefore, that despite the apparent requirement for the presence of the gufA promoter seen above, in other species this gene may either be absent or elsewhere on the chromosome. If this were the case, PCR would not be successful.

A better approach, perhaps, would be to use primers that were homologous to the open reading frames of carO and carR. Evidence is presented in a later chapter demonstrating the absolute requirement for the maintenance of translational coupling between these two genes. We would expect, therefore, carO and carR to be encoded on the same region of the chromosome if the species used the same regulatory mechanism believed to occur in M. xanthus. This approach would obviously not yield any information about the promoter of these genes. The amplified DNA could, however, be used in further experiments to probe the chromosomal DNA for a larger fragment that encoded the whole region.

3.4.2 An approach based upon the attB site of M. xanthus:

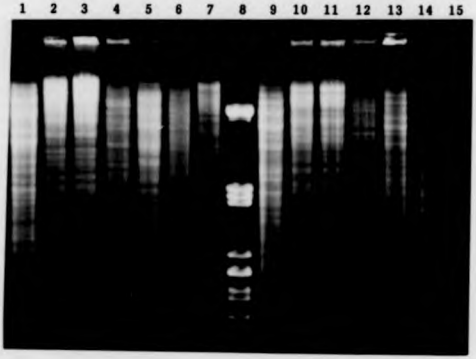
The integration of fragments into the Mx8 attachment site, has enabled the determination of the minimum region required for light-inducible promoter activity. In addition, and as described in later chapters, this recombination system is useful in the integration of fragments in other QUALITATIVE experiments. For a number of reasons, however, use of this approach in any QUANTITATIVE analysis of the promoter activity would be flawed.

The insertion into the chromosome, of the promoter probe, pSJM103 and any of its derivatives, appears to extend significantly the doubling times of the resulting strains (data not shown). Such a growth effect does not occur when pDAH274 has integrated into the carQRS region by homologous recombination. This would seem to indicate, either that the site of insertion is important in cell growth, or, more likely perhaps, that other phage related (and potentially deleterious) factors are encoded by the Mx8 attP fragment. No information is available as to the nature of any other genes encoded by this fragment.

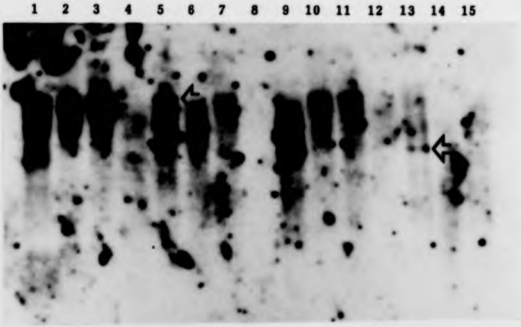
Evidence has recently been reported for the presence of more than one attB site upon the M. xanthus chromosome (Kaplan H., pers. comm.). Without knowing the position of insertion of different fragments, the comparison of the level of activity of different promoters becomes more difficult. In addition, the pattern of expression of the promoter inserted at this site appears quite different from that expected. The background activity is higher and the maximal activity is lower after exposure to light, when compared to wild-type, again raising doubts over interpretation of results.

Figure 3.11 Restriction digest and blot of chromosomal DNA from different species of myxobacteria: DNA was isolated from M. xanthus (tracks 1 & 9), Stigmatella aurantiaca strain DW4 (2 & 10), S. aurantiaca strain Sga1 (3 & 11), Cystobacter fuscus (4 & 12), M. virescens (5 & 13), M. fulvus (6 & 14) and M. stipitatus (7 & 15). DNA was digested with PstI (tracks 1 to 7) and SalI (tracks 9 to 15) and run on an agarose gel (panel A). Track 8 was lambda DNA digested with PstI. A Southern blot of this gel was probed with the carQRS insert of plasmid pDAH230. The resulting autoradiograph is shown in panel B. Positive signals corresponding to approximately 30Kb and 8Kb (in tracks 5 and 13 respectively; arrowed) are of the same size as those found in the M. xanthus control DNA and are found only in DNA isolated from M. virescens.

(A)



(B)



Chapter 4.

Characterisation of carQRS Mutant Strains.

4.1 Cloning of carQRS region of six mutant strains:

4.1.1 Myxococcus xanthus strains used in this work:

The carQRS regions of six carotenogenic mutant strains were cloned and subsequently sequenced. These strains display a variety of carotenogenic phenotypes (Figure 4.1) and were isolated in a variety of different ways. In each case, co-transduction frequencies with known transposon insertions had previously indicated that the mutations responsible for each phenotype were associated with the carQRS region (Murillo F., pers. comm.). Three Car^C strains were analysed in this study. One of these, strain MR151, was isolated as a red colony following UV mutagenesis of the wild-type strain, DK1050 (Martinez-Laborda et al., 1986). This mutation has been named carR3 (Balsalobre et al., 1987).

Three strains, MR446, MR448 and MR466, were each isolated from the Car^- strain, MR401 (Balsalobre et al., 1987) in another laboratory (Murillo F., pers. comm.). This strain contains a Tn*5* lac insertion (carB2) in the carB region such that production of β-galactosidase from the inserted lacZ gene is under the control of the carB promoter and is light-inducible. Following mutagenesis, three colonies were isolated as constitutive producers of β-galactosidase. The mutations that had been induced in each of these three strains appeared to render expression of the carB promoter, light independent. On replacement of the original Tn*5* lac insertion by co-transduction of the wild-type DNA with Tn*5*∩MR134 (Martinez-Laborda et al., 1986), two of the resulting strains, MR446 and MR448, were found to be Car^C. In contrast, the third strain, MR466, was found to be Car^+. The mutation encoded by this strain has been named car-10.

Finally, the carQRS regions of two Car$^-$ strains, MR132 and MR171 (Martinez-Laborda and Murillo, 1989) were analysed. These two strains were isolated as spontaneous yellow colonies arising from two Car^c strains, DK406 and DK8000 respectively (both parental strains carry the carR4 allele). The spontaneous mutations that occurred within these two strains have been named, car-11 and car-13 respectively.

4.1.2 Cloning of the carQRS regions:

The chromosomal DNA of five of these strains (the exception was MR406) were subjected to Southern blot analysis in order to confirm the expected sizes of each of the carQRS regions. This was an important consideration because knowledge of the size of the region was used in the cloning strategy adopted (see below). The chromosomal DNAs of the five strains were digested to completion with MluI and the resulting fragments separated by agarose gel electrophoresis. The similarly digested DNAs of strains DK101 (wild-type) and MR135 (a strain in which the carQRS region has been deleted) were also run on the gel. The resulting blot of this gel was probed with the 5200bp SohI/XhoI fragment of pDAH247 (Hodgson D. pers. comm.) containing the carQRS region. The autoradiograph of this blot indicated that each of the six mutant strains contained a carQRS region of the same size as the wild-type strain DK101 (data not shown).

In order to clone the carQRS regions of the six mutant strains, we followed the cloning strategy previously used in the analysis of other mutant strains (Figure 4.2; McGowan, 1989).

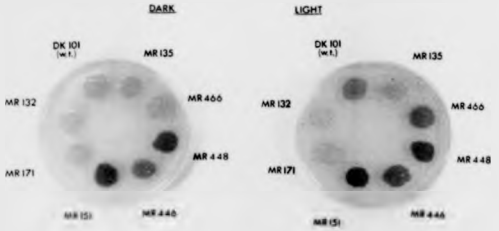
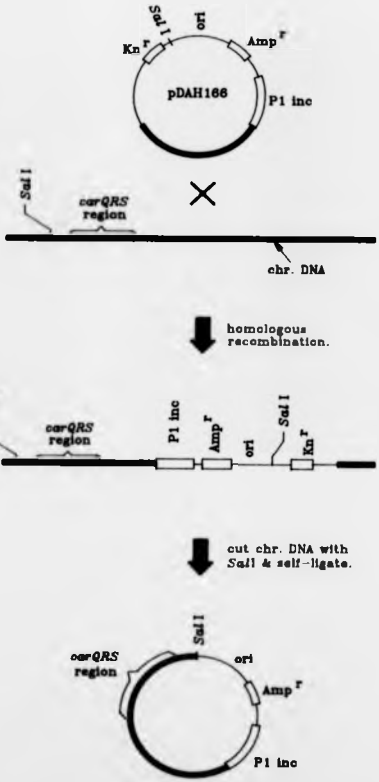


Figure 4.1 The carotenogenic phenotype of six mutant strains: Strains MR151, MR446 and MR448 are Car^C, producing carotenoids in both the light and the dark. Strains MR132 and MR171 are Car^-. Strain MR466 is Car^+, but encodes a mutation within the carQRS region that causes constitutive expression of carB. The strains may be compared with both the wild-type strain, DK101 and the Car^- strain, MR135 which is known to have undergone a deletion of the carQRS region.

Figure 4.2 Cloning strategy used to clone the carQRS regions of six mutant strains: The plasmid, pDAH166 encodes part of the M. xanthus chromosome near to the carQRS region. It integrates into the chromosome by homologous recombination. This is followed by restriction endonuclease digestion by SalI and isolation of part of the chromosome encoding the plasmid origin of replication and the host carQRS region. Self-ligation of this fragment enables its recovery by the transformation of a suitable strain of E. coli.


Sal I
ori
Kn r
Amp r
pDAH166
P1 inc
Sal I
carQRS region
chr. DNA
homologous recombination.
Sal I
carQRS region
P1 inc
Amp r
ori
Sal I
Kn r
cut chr. DNA with Sal I & self-ligate.
Sal I
carQRS region
ori
Amp r
P1 inc

The plasmid, pDAH166 encodes part of the *M. xanthus* chromosome near to the *carQRS* region and was employed to transduce each of the six strains using P1. The plasmid was able to integrate into the chromosome by homologous recombination. Since the plasmid does not encode any of the *carQRS* region, it is not able to gene convert the mutation within any of the *carQRS* genes upon integration.

The next stage in the cloning procedure involved digestion of the chromosomal DNA of each of the resulting strains with *Sal*I. This enzyme digests the chromsomal DNA once within the integrated plasmid DNA and again on the other side of the *carQRS* region. Thus, digestion results in a fragment of 14Kb that encodes both the *carQRS* region, an ampicilin resistance gene and the origin of replication of the plasmid. Isolation of this fragment, followed by self-ligation and transformation of a suitable *E. coli* strain, should result in a plasmid encoding the *carQRS* region of the original host.

Since it was obviously important that the integration of the plasmid had proceded as expected, Southern blot analysis of five of the resulting strains was carried out (again, strain MR466 not included). The resulting autoradiograph showed that in each of these strains, digestion with *Sal*I yielded the expected 14Kb insert (Figure 4.3).

Using this procedure, we were able to clone the *carQRS* regions of each of the six mutant strains into six plasmids (Table 4.1).

Figure 4.3 Autoradiograph of the chromosomal DNAs of five mutant strains each transduced with pDAH166: DNA was isolated from strains MR132, MR151, MR171, MR446 and MR448, each transduced with the plasmid, pDAH166 (lanes 2 to 6). The chromosomal DNAs were digested to completion with SalI and run on an agarose gel (panel A). The plasmid, pDAH333 (McGowan, 1989) linearised with PstI, was also run on the gel as a control (lane 1). A blot of this gel was probed with the 3300bp MluI/PstI fragment of pDAH333. The resulting autoradiograph shows each strain to have a copy of the plasmid integrated into the chromosome (panel B).

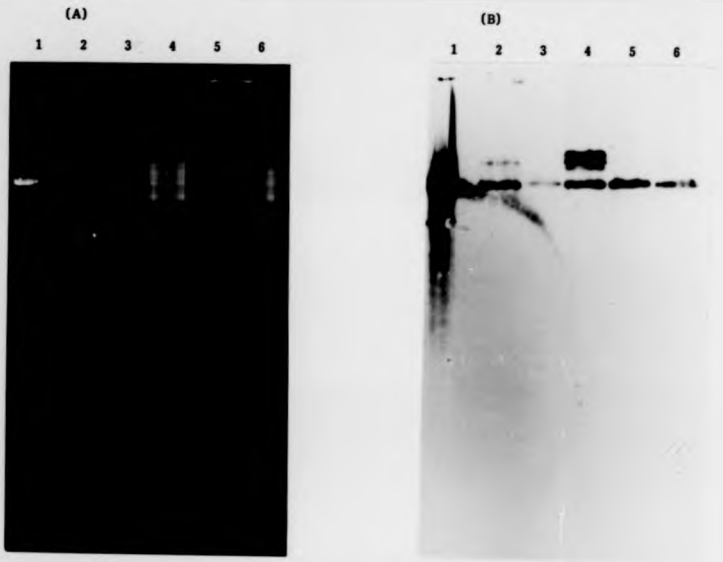

(A)
1 2 3 4 5 6
(B)
1 2 3 4 5 6

Table 4.1 Mutant carotenogenic strains and the plasmids in which the carQRS region of each has been cloned:

Strain	Plasmid
MR132	pSJM110
MR151	pSJM111
MR171	pSJM112
MR446	pSJM113
MR448	pSJM114
MR466	pSJM115

4.2 DNA sequence analysis of carQRS region of mutant strains:

4.2.1 Location of mutations:

Using the known restriction map of the carQRS region, a variety of fragments from each plasmid were in turn cloned into M13, mp18 or mp19 and sequenced. With one exception, the DNA sequence of all of the carQRS genes, as well as the light-inducible promoter region, from each of the strains was examined. The carR gene only of MR151 was sequenced. In each case, a mutation within the carQRS region encoded by the plasmids was found (Figures 4.4 - 4.8).

The three Car^0 phenotypes of strains MR151, MR446 and MR448, were each found to be caused by a single base change within the carR gene. A

transition in which the guanine at position 1782 has been replaced by adenine was found within the *carR* gene of strain MR151 (Figure 4.4). This mutation leads to an alteration of the primary amino acid sequence of the CarR protein, in which glutamic acid is replaced by lysine.

In strain MR446, the same transition event, this time at position 1851, results in the amino acid, glycine being replaced by arginine (Figure 4.5). In strain MR448, a guanine to adenine transition was found at position 1970 (Figure 4.6). This is the same mutational event that was found to have occured in the Car^C strain DK2834 (*carR6* mutation; McGowan, 1989). It results in premature termination of translation of *carR*, as the TGG codon normally encoding tryptophan has become altered to encode the stop codon, TGA.

The two Car^- strains, MR132 and MR171, were both shown to have undergone a small deletion of the chromosome within the *carQ* gene. A 9bp deletion was discovered within the MR132 *carQ* gene, between positions 1377 and 1387 (Figure 4.7). Similarly, a 12bp deletion between positions 1646 and 1659 was found in the *carQ* gene of MR171 (Figure 4.8). The *carR4* mutation of the parental strains at position 2354, was also found within the *carR* gene of both these two strains (data not shown).

Finally, a point mutation within the *carS* gene at position 2611, was discovered in the MR466 derived DNA (Figure 4.9). Once again, this is a transition event involving replacement of guanine with adenine. In addition, this mutation resembles that of strain MR448, in that a codon for the amino acid tryptophan TGG, has become altered to encode the stop codon, TGA. The mutated gene therefore, encodes a truncated form of the CarS protein of only 86 amino acids, compared with the wild-type form of 111 amino acids.

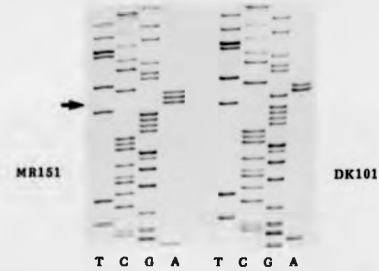


Figure 4.4 Autoradiograph of DNA sequencing gel showing part of the carR gene sequence from strains MR151 and DK101: The guanine ("G") in the wild-type sequence of strain DK101 has been replaced by adenine ("A" - arrowed) in strain MR151.

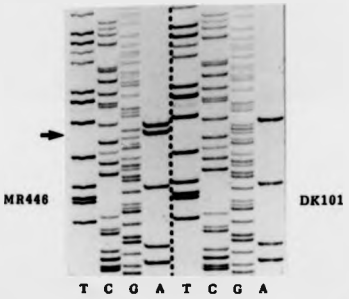


Figure 4.5 Autoradiograph of DNA sequencing gel showing part of the carR gene sequence from strains MR446 and DK101: The guanine ("G") in the wild-type sequence of strain DK101 has been replaced by adenine ("A" - arrowed) in strain MR446.

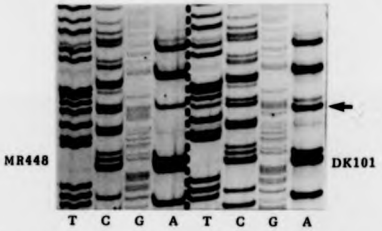


Figure 4.6 Autoradiograph of DNA sequencing gel showing part of the carR gene sequence from strains MR448 and DK101: The guanine ("G") in the wild-type sequence has been replaced by adenine ("A") in strain MR448.

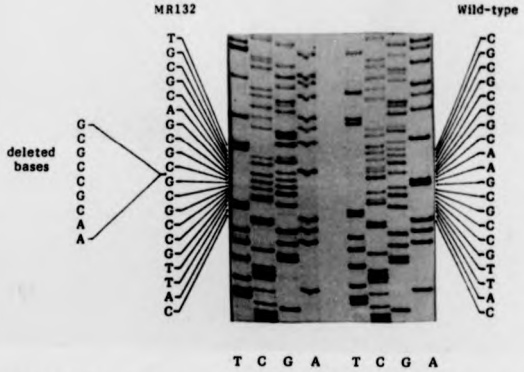


Figure 4.7 Autoradiograph of DNA sequencing gel showing part of the carQ gene sequence from strains MR132 and DK101: Nine base pairs of the chromosome have been deleted within the carQ gene of strain MR132.

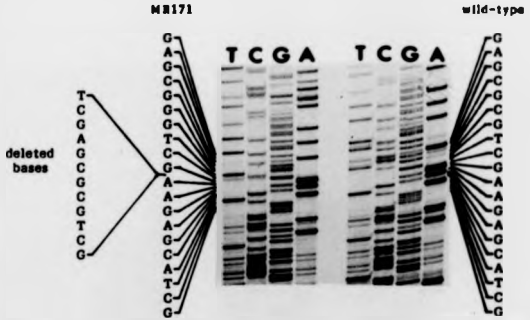


Figure 4.8 Autoradiograph of DNA sequencing gel showing part of the carQ gene sequence from strains MR171 and DK101: Twelve base pairs of the chromosome have been deleted within the carQ gene of strain MR171 when compared to the wild-type DNA sequence.

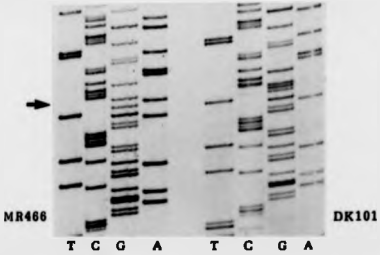


Figure 4.9 Autoradiograph of DNA sequencing gel showing part of the carS gene sequence from strains MR466 and DK101: The guanine ("G") in the wild-type sequence has been replaced by adenine ("A") in strain MR466.

4.3 Analysis of results and discussion:

4.3.1 Mutations within carR:

Before this work had been carried out, we perhaps expected to find that the mutations in strains MR151, MR446 and MR448 giving rise to the Car^C phenotypes, were within the carR gene. This was because the chromosomes of three other Car^C strains had all previously been shown to have undergone a point mutation within this gene (McGowan, 1988).

The analyses of the chromosomes of these strains are consistent with the hypothesis that the carR gene product negatively regulates the activity of P^{QRS}. Inactivation of this protein by mutation, leads to unregulated constitutive activity of P^{QRS} and to the observed Car^C phenotype. The function of this protein would appear to be particularly sensitive to peturbation of its primary amino acid sequence. We now know of six point mutations (although two of these are the same) at different positions throughout the carR gene, that each give rise to a Car^C phenotype. A possible mechanism by which the wild-type CarR protein exerts this negative influence over the light-inducible promoter is discussed later in this study.

We propose to name the two mutations encoded by the strains MR446 and MR448, carR7 and carR8 respectively.

4.3.2 Mutations within carO:

The only mutation previously known to occur at the carORS region and to give rise to a Car^- phenotype had occured in strain MR135

(Martinez-Laborda and Murillo, 1986). The chromosome of this strain has been shown to have undergone a deletion of some 15Kb, in which the whole of the carQRS region has been removed (Hodgson D., pers. comm.). Clearly, from the Southern blot analysis data described above, this had not happened to the chromosomes of either strain MR132 or strain MR171.

In both these strains, the mutation that would appear to give rise to the Car$^-$ phenotype, has again occurred by a deletion event involving the carQRS region. In these two strains, however, the deletions are of only 8bp and 12bp and have taken place within a gene for which no function has yet been ascribed, carQ. Because we now know their location, we propose to rename the mutations within strains MR132 and MR171, carQ1 and carQ2 respectively. Further work to determine the function of the carQ gene product is described in a later chapter (Chapter 5).

The two deletions within the carQ gene both occurred spontaneously. If the context of the deleted regions is examined, a possible mechanism for their formation may be deduced. The deleted DNA in each case is found to have homology with DNA either immediately upstream or immediately downstream of the site of deletion (Figure 4.10). In MR132, 7bp of the 8bp deletion are directly repeated upstream. In strain MR171, this homology is more striking, with 11bp of the 12bp deletion directly repeated immediately downstream of the site of deletion.

The presence of these small direct repeats adjacent to the site of deletion in both strains, suggests that they are involved in the generation of the deletions. Such direct repeats have previously been implicated in this regard and a model for the deletion of DNA involving direct repeats has been developed (Efstratiadis et al., 1980).

As outlined in Figure 4.11, the deletions are believed to occur as the

chromosome is replicated. As the replication fork moves through the region and the two strands are separated, mismatches between the two strands may be formed between the repeats. The downstream repeat, for example, of the "upper" DNA strand, may base-pair with the upstream repeat of the "lower" DNA strand. This would result in the formation of a single stranded loop in the "upper" strand encoding the upstream direct repeat. Such a loop may be recognised by DNA repair enzymes and can be removed. This event would be followed by religation of the ends of the strand involved. Progeny containing a chromosome derived from this strand would encode only the downstream direct repeat of the parental chromosome.

4.3.3 A mutation within the carS gene:

The mutation that occurred within the chromosome of strain MR466 to give rise to a $carB^C$ phenotype, was located in the carS gene. This is consistent with the hypothesis described in Chapter 1, that the carS gene product encodes an activator of carotenogenesis. This activator is normally only expressed upon light-induction.

Two questions remain, however, if we accept this hypothesis. Firstly, how does the truncated version of the CarS protein activate the carB gene when expressed from the carQBS operon in the dark only at very low levels? Secondly, if as now seems the case, an effective activator of the car-related genes is now present, why is the strain not Car^O? These points are addressed in Chapter 7 following an investigation into the nature of the activation of promoters at carB and carC.

Figure 4.10 DNA sequence of the carQ gene and the positions of the mutations in strains MR132 and MR171: The two pairs of direct repeats within the DNA sequence of carQ have been boxed. The downstream direct repeat of the first pair and the upstream direct repeat of the second pair have been deleted in strains MR132 and MR171 respectively (as indicated).

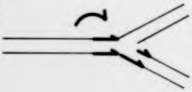
Figure 4.11 Model for the generation of deletions involving directly repeated DNA sequences: (adapted from Efstratiadis et al., 1980.) As the replication fork moves through the region during chromosome replication, the two strands are separated. Mismatches between the two strands may subsequently be formed between the repeats. In the model shown, the downstream repeat of the "upper" DNA strand, may base-pair with the upstream repeat of the "lower" DNA strand. This would result in the formation of a single stranded loop in the "upper" strand encoding the upstream direct repeat. Such a loop may be recognised by DNA repair enzymes, be removed, and the two ends of the strand involved religated. Progeny containing a chromosome derived from this strand would encode only the downstream direct repeat of the parental chromosome.

1.

2.

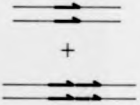
3.

4.



5.

6.



4.4 Further analysis of carQRS region of strain MR466:

4.4.1 Does the carS mutation cause the carB^{C} phenotype of strain MR466?

The results of separate experiments involving transposon linkage data, had previously indicated the likely location of the mutation responsible for the phenotype of MR466, to be within gufA (Murillo F., pers. comm.). An experiment was carried out, therefore, in order to determine whether the mutation we had found by sequence analysis was responsible for the observed carB^{C} phenotype. We cloned the 897bp fragment from the SacI site at position 2232 to the XhoI site at position 3128 from pSJM115 into the plasmid pDAH142 (Hodgson D., pers. comm.). This fragment encodes part of carB and all of carS of the carQRS operon of MR466. It does not encode either P^{QRS}, or the genes carQ and gufA. The resulting plasmid was named pSJM127.

Using P1, this plasmid was used to transduce the strain MR418 (Martinez-Laborda *et al.*, 1990). This strain, like MR401 from which it was derived, contains a Tn*5* *lac* insertion (carB2) in the carB region. The production of β-galactosidase from the inserted, promoterless lacZ gene is, therefore, under the control of the carB promoter. The kanamycin resistance gene of the integrated Tn*5* *lac* of MR401 has been replaced *in situ* by the oxytetracycline resistance gene of Tn*5*-132. Thus, resistance to kanamycin could be used in the selection of strain MR418::pSJM127.

When the plasmid pSJM127 integrated into the chromosome by homologous recombination at the carQRS region, expression of the incoming MR466 carS gene could become driven by the light-inducible promoter (depending upon the position of the crossover and assuming no gene con-

version - see Figure 4.12). If the *carB*^{C} phenotype of MR466 was indeed caused by the mutation within the *carS* gene, this recombination event would result in constitutive expression of beta-galactosidase from the *carB* fused *lacZ* gene.

By including the chromogenic substrate, Xgal within the media, the transductants were screened directly for expression of *lacZ*. Several of the resulting transductants were found to express this gene constitutively (data not shown) and we conclude that the point mutation already described is responsible for the *carB*^{C} phenotype of MR466. We propose, therefore, to rename this mutation, *carS1*.

4.4.2 Is the *carS1* mutation dominant or recessive?

As already described, the product of the *carS* gene is believed to exert an influence over the transcription of genes within the *carB* region. The determination of the dominance or otherwise of the *carS1* mutation over the wild-type allele would indicate whether this gene encodes an activator or a repressor of promoters located within *carB*.

One approach that was considered in order to resolve this question, was to use the plasmid pSJM112 to transduce strain DK101 and to simply observe the phenotype of the resulting colonies. The process of homologous recombination during integration of the plasmid, however, was expected in some instances to lead to gene conversion of either of the *carS* alleles. Such gene conversion would make interpretation of the results difficult and this experiment, therefore, was not carried out.

Instead, we decided to clone the *carQRS* region of MR466 into a plasmid derived from the modified promoter probe, pSJM103 (Chapter 3).

This plasmid encodes the site specific recombination system of Mx8 and it has been reported that integration using this system, leads to a much reduced frequency of gene conversion (Li and Shimketa, 1988).

We digested pSJM115 with XhoI and ScaI and isolated the resulting 10.2Kb fragment. This fragment encodes the carQRS region of MR488 and the incompatibility region of P1. A 8.7Kb fragment of pSJM103 was isolated following digestion of this plasmid with BamHI. This fragment encodes the plasmid origin of replication and a kanamycin resistance gene, as well as the site specific recombination system of Mx8. It does not contain the promoterless lacZ gene of pSJM103. Both fragments were blunt ended using T4 DNA polymerase and the Klenow fragment of DNA polymerase I and were subsequently ligated. The resulting plasmid named pSJM149 was used to transduce via P1, strain MR418.

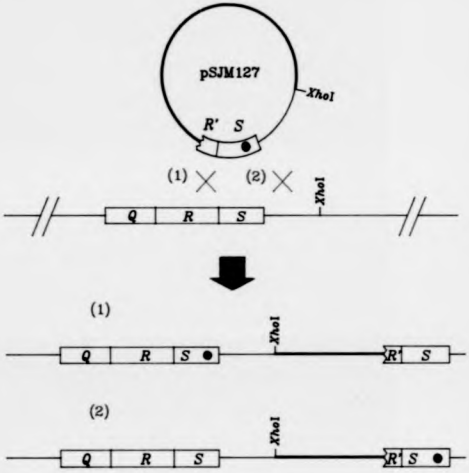
Again, Xgal was included in the media to directly screen the transductants for expression of lacZ. The majority of the resulting transductants were found to express this gene constitutively (data not shown). The small minority of colonies, at less than 2%, are assumed to be the result of gene conversion of the incoming carS1 allele by the wild-type allele during homologous recombination at the carQRS region.

The result of this experiment shows that the carS1 allele is dominant to the wild-type carS allele. Rather than encoding a repressor, the carS gene would seem, therefore, to encode an activator of the genes at carB. This result, however, leaves unanswered further questions about the carS gene product. Most notably, it is difficult to imagine how a truncated protein, with between a quarter and a third of its carboxy-terminal end absent, can be a more effective activator than its full length counterpart. The answer to this problem undoubtedly lies in an understanding of the

mechanism of activation by the CarS protein.

As previously described, the derived amino acid sequence of the *carS* gene shows no homology to any known protein (McGowan, 1988). In particular, it does not exhibit the DNA binding domains that we might expect to be associated with its function. Further work into the activation of genes at *carB* by the interaction between the *carS* gene product and the *carA* region (or any products thereof), might be expected to reveal an answer to this particular problem.

Figure 4.12 Integration of pSJM127 into the chromosome of M. xanthus strain MR418: During integration of the plasmid into the chromosome by homologous recombination at the carQRS region, the crossover can take place on either side of the mutation within carS (●). A crossover at position 1 will lead to expression of the incoming mutated carS allele by P^{QRS}. If the incoming point mutation is responsible for the $carB^{C}$ phenotype of MR466, then in strain MR418 where the carB promoter is fused to a promoterless lacZ gene, a crossover in this position would lead to constitutive expression of β-galactosidase.



Chapter 5.

carQ Gene Function.

5.1 Introduction:

The light-inducible promoter of the carQRS operon cannot function in vivo if all three carQRS genes have been removed (Robson, 1992). This was demonstrated by inserting the site specific recombination system of bacteriophage Mx8, into the promoter probe pDAH288 (where P^{QRS} alone is upstream of a promoterless lacZ gene), to generate plasmid pPR108. The activity of the light-inducible promoter was then assayed in strain MR135 (a strain in which the carQRS region has been deleted) and found to be negligible.

We had previously assumed that the carR gene product was a negative regulator of the promoter (McGowan, 1989) and that the promoter in the absence of carR would exhibit unregulated constitutive activity. The above result, however, whilst not contradicting the carR/negative regulator hypothesis, seemed to imply that one of the other two genes within the region was responsible for activation of P^{QRS}.

We have already shown that a viable carQ gene is required for carotenogenesis (Section 4.2.1). In order to determine, therefore, whether the carQ gene was required specifically for P^{QRS} activity, we assayed the activity of P^{QRS} in the presence of carQ alone.

5.2 Construction of carQ/lacZ fusion:

In order to assay the activity of the promoter and carQ gene in vivo, we made use of a plasmid constructed during sequencing of the carQRS region (McGowan, 1989). Before sequencing began, a series of nested deletions within the carQRS region had been constructed using the enzyme

exonuclease III. One member of this deletion series contained a fragment from which both the carR and carS genes had been deleted. The remaining DNA, in a plasmid named pSJM3, contained the light-inducible promoter region and carQ gene alone.

The promoter/carQ DNA fragment was isolated from the pIC19R vector by digestion of pSJM3 with EcoRI and agarose gel electrophoresis. It was subsequently cloned in both orientations into bacteriophage M13 mp18 and the integrative promoter probe pSJM103, both digested with EcoRI.

The insertion into M13 was used to sequence the ends of the fragment. The resulting sequence showed that the fragment was 884bp long and confirmed that it contained the light-inducible promoter and the whole of carQ (Figure 5.1). Moreover it also showed that only the codons for the first five amino acids of carR were present before the sequence of part of the pIC19R polylinker from the original construct.

The two plasmids resulting from insertion of the fragment into pSJM103 were named pSJM107 and pSJM116. The orientation of the fragment within pSJM107 enabled P^{QRS} to drive expression of the promoterless lacZ gene on the probe. In the opposite orientation in pSJM116, expression of the lacZ gene was driven by the gufA promoter that was also present on the fragment.

5.3 Activity of promoter + carQ gene:

The activity of P^{QRS} with the carQ gene was assayed in vivo in a variety of genetic backgrounds. To this end, the strains MB135, DK101, MB132 and MB522 were transduced with P1 packaged pSJM107. It was found that the Car phenotypes of the resulting strains varied (Figure 5.2

and summarised in Table 5.1) and that in each case, the phenotype was the same if pSJM116 was used in the transduction instead of pSJM107 (data not shown).

In addition, the levels of β-galactosidase produced by the lacZ gene in these strains was assayed in both the light and the dark (Figures 5.3, 5.4 and 5.5).

Table 5.1 Effect on regulation of carotenoid production of a variety of strains following transduction with pSJM107 or pSJM116:

Strain	Original Car phenotype	After transduction with pSJM107/pSJM116:	
		Colour	Car phenotype
MB135	Car^-	yellow	Car^-
DK101	Car^+	red	Car^C
MB132	Car^-	red	Car^C
MB522	Car^-	red or yellow	Car^C

```
        10         20         30         40         50         60         70
GAATTCATCG ATATCTAGAT CTCGAGCTCC GAGGTGACGA GCACGGCCAG CGCCCCCAGT CCCGTCGCCG

        80         90        100        110        120        130        140
TCCCCCCCAG CAGCCTTCCC ACCAACCCCG CCCCCATGCC TTCGATCCTC CAATCGTCCA CCCGTCCGAG

       150        160        170        180        190        200        210
GTCCCTCCGC GCCCAACACT AACCCCCTCG CCCTCCACGG CAGGACCGCA TCCTCCTCGC GTTCGCAACC

       220        230        240        250        260        270        280
CCGTGACTTT CCAGAGCTTT CCTCACCGAA CCTTGAGAAG CCCGAGCCCC CGAAACACTT TCCCAGGTCG

       290        300        310        320        330        340        350
CCCGTAGAGG AGTCCGGTGA TGCCGAGCCA AACCGACGAA GCACTCAGCG AACCCTTTCG CGACCCACCA
                                                  MetG luArgPheAr gAspGlyAla

       360        370        380        390        400        410        420
CAGGACCCCT TCGACGACCT CTTCCCCCCC CACCCCCCCC GCCTCCAACG CTTCCTCCCC CCGACCCCCC
GlnAspAlaP heGluAspLe uPheAlaArg HisAlaProA rgValGlnGl yPheLeuAla ArgMetValA

       430        440        450        460        470        480        490
GCAACGCCCC GCTCCCCCAA CACCTCCTCC AGCCCACCTT CTTCTCCCTC ATCCCCTCCC CCCCCCCCTA
rgAsnGlyAl aLeuAlaGlu AspLeuLeuG lnAlaThrPh eLeuSerVal IleArgSerA rgGlyArgTy

       500        510        520        530        540        550        560
CGACCCCCCC ACCCCCTTCA TCCCCTCCCT CATCACCATT GCCCCGAACG CCCCCCCCCA CCCCTTCACG
rGluProGly ThrArgPhel leProTrpLe uMetThrIle AlaAlaAsnA laAlaArgAs pAlaLeuArg

       570        580        590        600        610        620        630
CATCACCCCC ACCTACACCC CTACCCCTCC CCCCACCACA CCCCCACCCC CCCATCCCCC GCCCCCCATG
HisGlnArgI isValAspAl aTyrAlaSer ArgGluAspT hrAlaThrPr oAlaSerAla AlaProAspA

       640        650        660        670        680        690        700
ACAGCCACCC GTCCCTCCCC ACCCACCTCC TCCACCCCTT CCACAACTCC CACCCCCACC ACCCTCAACC
spSerAspPr oSerLeuArg ArgHisLeuL euAspAlaLe uGlnGlnLeu HisProAspH isArgGluAl

       710        720        730        740        750        760        770
CCTCCTCCTC ACCAACCTCG ACCCCTCCTC CTTCCACCAC ATTCCCCCCC TCCCCCCCAT CACCCCTCCG
aValValLeu SerLysValG luGlyTrpSe rPheGluGlu IleGlyAlaL euArgGlyIl eSerProGly

       780        790        800        810        820        830        840
CCCCCCCCCC TCCCACCCCA CCCCCCCCAC CACAACCTCC CCCACCTCCT CCCCCACCTC CACCTCCACG
AlaAlaArgL euArgAlaHi sArgGlyTyr GluLysLeuA rgGluLeuLe uGlyGluLeu GluLeuGluV

       850        860        870
TCCCCCCACG AACCCACCGA TCGTCCACCG ATCCCCCCGAATTC
alAlaArgEND
      MetLysProProMet - carR
```

Figure 5.1 DNA sequence of the light-inducible promoter and carQ gene fragment of pSIM3: Only five codons of the carR gene remain. Flanking EcoRI restriction endonuclease sites have been underlined.

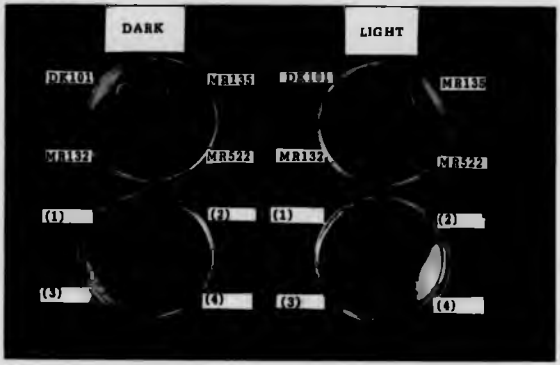
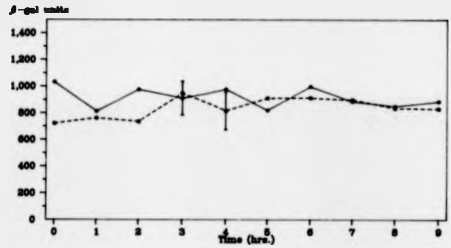


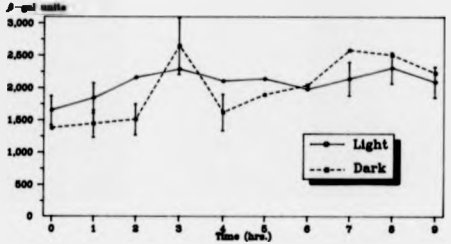
Figure 5.2 Car phenotypes of four strains following transduction with pSJM107: The wild-type strain DK101 and the three Car$^-$ strains, MR135, MR132 and MR522 were each transduced with pSJM107, encoding p^{QRS} and carQ (number 1 = DK101::pSJM107; number 2 = MR135::pSJM107; number 3 = MR132::pSJM107; number 4 = MR522::pSJM107). After transduction, MR135 remained yellow in both the light and the dark and, therefore, remains Car$^-$. Each of the other three strains became red with the accumulation of carotenoids in both the light and the dark (Car^C).

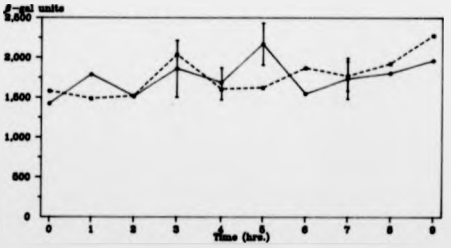
Figure 5.3 β-galactosidase activity of strain MR135::pSJM107: Strain MR135, a strain in which the wild-type carQRS region had been deleted, was transduced with pSJM107. The plasmid encodes P^{QRS} which drives expression of carQ and lacZ and integrates at the Mx8 attB site on the chromosome.

Figure 5.4 β-galactosidase activity of strain DK101::pSJM107: The wild-type strain DK101 was transduced with pSJM107. The plasmid encodes P^{QRS} which drives expression of carQ and lacZ and integrates into the chromosome at attB.

Figure 5.5 β-galactosidase activity of strain MR132::pSJM107: Strain MR132, a strain in which a small region of the carQ gene had been deleted (Chapter 4), was transduced with pSJM107. The plasmid encodes P^{QRS} which drives expression of carQ and lacZ and integrates into the chromosome at attB.


β-gal units
1,400
1,200
1,000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9
Time (hrs.)


β-gal units
3,000
2,500
2,000
1,500
1,000
500
0
Light
Dark
0 1 2 3 4 5 6 7 8 9
Time (hrs.)


β-gal units
2,500
2,000
1,500
1,000
500
0
0 1 2 3 4 5 6 7 8 9
Time (hrs.)

5.4 Gene carO is required for light-inducible promoter expression:

5.4.1 MR135::pSJM107 exhibits constitutive promoter activity and is Car⁻:

The strain MR135 has undergone a large deletion of its chromosome and no longer contains any of the carQRS region. Following transduction with pSJM107, the results of the β-galactosidase assays on strain MR135::pSJM107, would appear to show that expression of the integrated P^{QRS} is constitutive at very high levels (Figure 5.3). This presumably also means that there are very high levels of the carO gene product also being expressed within the cell (the carO gene is encoded between the light-inducible promoter and the lacZ gene).

In a similar experiment in which the same strain was transduced with the promoter alone, no expression of the integrated promoter could be detected (Robson, 1992; Section 5.1). Taken together, these two results show that the carO gene, and presumably, therefore, its gene product, is absolutely required for expression of the light-inducible promoter. The carO gene product would appear to be an activator of P^{QRS} and in strain MR135::pSJM107, this activation has become constitutive. The carO gene product activates P^{QRS} to cause its own expression and is, therefore, engaged in positive feedback in the absence of the carR gene product.

The strain MR135::pSJM107 remains Car⁻, unable to produce carotenoids in either the light or the dark (Figure 5.2). It would seem, therefore, that the carO gene product is not an activator of the carotenoid biosynthetic genes at carB and carC. This would agree with the results of experiments described in Section 1.7.4, where it was proposed that the carS gene is likely to encode such an activator.

5.4.2 DK101::pSJM107 exhibits constitutive promoter activity and is Car^C:

Further confirmation of the above is provided by the Car phenotype exhibited by the strain DK101::pSJM107. The original strain, DK101, is wild-type in both its *car* genotype and ability to produce carotenoids in response to light. When transduced with pSJM107, however, the strain produces carotenoids constitutively to remain red in both the light and the dark (Figure 5.2). This Car^C phenotype may be explained by examination of the *lacZ* assays of this strain (Figure 5.4). Again, in this strain, expression from the promoter is constitutive at very high levels in both the light and the dark.

We have already demonstrated that the *carO* gene product acts in an unregulated, positive manner on P^{ORS} in strain MR135::pSJM107 to promote its own constitutive expression. Similarly, in strain DK101::pSJM107, the very high levels of the *carO* gene product are caused by this cis-acting positive effect on the newly integrated promoter.

In addition, the high cellular levels of *carO* have a trans-acting effect on the promoter at the wild-type *carORS* operon that is also present in the DK101 chromosome. The *carO* gene product presumably acts to promote constitutive expression of this promoter and, therefore, constitutive expression from the operon of an activator of the genes at *carB* and *carC*. Continuous activation of these genes would lead to the Car^C phenotype observed. This in turn leads us to conclude that *carS* is likely to encode an activator of these genes.

5.5 A model for the regulation of P^{QRS} activity:

5.5.1 Development of a model:

Removal of the *carR* gene in strain MR135::pSJM107 results in constitutive expression of P^{QRS}. As stated above, this would seem to indicate that *carQ* encodes an activator of P^{QRS} and that this activation is regulated by the *carR* gene product. In the dark, therefore, the CarR protein is able to inhibit the activity of the CarQ activator. This ability is presumably lost upon reception of a light stimulus.

The CarR protein is unlikely to act as a "classic" DNA binding repressor because it has no homology with any known DNA binding proteins and is believed to be membrane associated (McGowan, 1988). Any model that is developed for the mechanism of light regulation of *carQRS*, must address, therefore, the means by which CarR is able to exert its negative influence over P^{QRS} and the ability of light to interfere with this process.

One system often used by bacteria for the regulation of response to an external signal is the "two component" system (Stock *et al.*, 1989) and indeed, one such system has already been reported in *M. xanthus* (*frz* genes; McBride *et al.*, 1989). Like the regulation of P^{QRS} described above, the two component system also involves two proteins: the often membrane bound histidine protein kinase and a response regulator. Whilst being analogous to this system, however, neither of the protein products of *carQ* and *carR* show any significant degree of homology with consensus amino acid sequences of two component proteins. Regulation of expression of P^{QRS}, therefore, does not appear to involve the protein phosphorylation mechanism thought to be inherent in these systems.

Instead, a separate model (developed by Hodgson D., pers. comm.) based upon the above results and the results of other experiments carried out within the group (described below in Sections 5.5.2 and 5.5.3) has been developed. The results of all of these experiments are summarised in Figure 5.8.

This model envisages the 1:1 ratio of *carQ* to *carR* genes, and hence their respective gene products, as being crucial to the correct functioning of the promoter. It had been noted that when this ratio is altered experimentally, the regulation of P^{QRS} is often also upset. In strain MR135::pSJM107, for example, only the activator gene, *carQ*, is present and the promoter fires constitutively. In strain DK101::pSJM107, there are two *carQ* genes to one *carR* regulator gene and again the promoter exhibits constitutive expression. Explanation of these and other results, is difficult using conventional models involving chemical modification of an activator of the promoter.

In order to explain these results in terms of a model, we believe that the observed 1:1 ratio between the *carQ* and *carR* genes argues for a close association between the two gene products. Since we believe that the CarR protein is a membrane protein (McGowan, 1989; Gorham H., pers. comm.), we would further argue that in the dark, each CarR protein sequesters one CarQ protein to the membrane. When light is "perceived" by the CarR protein, it "releases" the CarQ protein, thus enabling the latter to activate P^{QRS}.

We can see that this light-inducible activation of the promoter will only occur when there is a 1:1 ratio of CarQ protein to CarR protein. If the cellular levels of CarQ are greater than those of CarR, then not all of the CarQ protein will be sequestered to the membrane. In this situation,

the remainder is free to cause activation of the light-inducible promoter and hence constitutive production of carotenoids by the cell.

5.5.2 carQ:carR = 2:1:

This "sequestration" model is supported by previous results achieved within the group and in particular those of experiments involving an alteration of the ratio of carQ genes to carR genes. One such occurred when a fragment of the chromosome from the SacI site at position 865 to the SacI site at position 2232, had been used to transduce the wild-type strain DK101. This fragment of DNA encodes P^{QRS}, carQ and a truncated form of carR (greater than three quarters of the carR gene remain). When integrated into the chromosome by homologous recombination, this fragment caused constitutive production of carotenoids. The result was previously explained by the truncated regulator encoded by the incoming carR gene being both unable to function correctly and dominant to the wild-type regulator which is also present.

Clearly, the result may now be more convincingly explained in terms of the ratio of carQ genes to carR genes. In this strain, there are two carQ genes to the one functional carR gene. As in strain DK101::pSJM107, this results in there being insufficient CarR protein within the cell to regulate by sequestration, all of the activating CarQ protein. This will cause positive feedback of the carQ gene product on the promoter resulting in over expression of all genes in the carQRS operon and concomitant constitutive production of carotenoids.

5.5.3 carQ:carR = 1:2:

Another experiment performed within the group resulted in a ratio of one carQ gene to two carR genes (Gorham H., pers. comm.). This was again achieved by transduction of the wild-type strain DK101 with a fragment of the chromosome containing the carQRS operon. Before transduction, the carQ gene encoded by this fragment had been inactivated by the insertion of a small fragment of DNA at the BglII site (position 1281; the carQ3 mutation). This fragment of DNA was designed to maintain the frame of the carQ gene and as such ensure normal translation of the coupled carR and carS genes. Upon transduction and integration into the chromosome, this fragment of DNA had no effect upon the wild-type Car phenotype of the cells.

We may again explain this result in terms of the above described sequestration model. In the dark, because there is a higher cellular level of CarR protein than functional CarQ protein, all CarQ protein will be sequestered to the membrane. This is analogous to the wild-type strain DK101 in the dark and, therefore, no expression from either carQRS operon is expected. No carotenoids are produced and the cells remain yellow in the dark.

Upon perception of light, the model states that none of the CarR protein present in the cell will be able to continue this sequestration in spite of its higher cellular concentration. All CarQ protein would, therefore, be released and available to activate the two carQRS operons. Light-induction of this strain would, therefore, result in an essentially wild-type production of carotenoids.

5.5.4 MR132::pSJM107:

This model also goes some way to explaining the result of the experiment involving transduction of the Car$^-$ strain MR132 with pSJM107. The Car$^-$ phenotype of strain MR132 is caused by a small deletion within the *carQ* gene (*carQ1*, Chapter 4) and, therefore, we might expect integration of a wild-type *carQ* gene to complement this mutation and to result in a wild-type Car phenotype. It has been shown, however, that the resulting strain, MR132::pSJM107, exhibits a Car^C phenotype (Figure 5.2).

In order to explain this apparent anomaly, we must examine the genotype of the original strain MR132. This strain was selected as a reversion of the Car^C phenotype of strain DK406 (Martinez-Laborda *et al*., 1986). The Car^C phenotype was caused by the *carR4* point mutation within the *carR* gene (McGowan, 1989) and this mutation remains within the *carQRS* operon of MR132 (Section 4.2.1). The effective genotype of MR132, like MR135, is *carQ*$^-$/*carR*$^-$.

We have demonstrated that integration of the *carQ* gene of pSJM107 into the chromosome of strain MR132, again causes constitutive expression of the light-inducible promoter (Figure 5.5). This expression is due to the absence of a functional *carR* gene and, therefore, unregulated activation of the promoter by the CarQ protein. Unlike MR135, however, at the *carQRS* operon present from the original strain MR132, this activation will also cause expression of *carS*. This gene is believed to encode an activator of carotenogenesis. Since this expression of *carS* occurs in both the light and the dark, the strain MR132::pSJM107 exhibits a Car^C phenotype.

5.5.5 MR522::pSJM107:

Strain MR522 is Car$^-$ and has a transposon inserted into the chromosome at a previously unknown locus, carD (Nicolas F. and Murillo F., pers. comm.). It was generated using transposon mutagenesis of a Car^c strain (a strain in which there is a mutation within the carR gene), followed by direct selection for the Car$^-$ phenotype. The carQRS operon in this strain was inactive under light-inducing conditions. The carD locus, therefore, was believed to be involved in either the mechanism of regulation of the operon, or at an earlier stage than this, for example, in the initial reception of the light signal.

Integration of the plasmid pSJM107, however, gave rise to a mixed population of colony phenotypes: some Car$^-$ (yellow) and some Car^c (red). Upon restreaking of colonies exhibiting the red, Car^c phenotype, a similar mixed population of colony phenotypes was observed. Yellow colonies, however, only ever gave rise to further yellow, Car$^-$ colonies. It is for this reason that the Car^c phenotype is believed to be the "true" phenotype resulting from integration of pSJM107. The Car$^-$ phenotype is believed to result from a subsequent, high frequency event, possibly a deletion of part of the chromosome, that results in loss of one of the carQ genes. In addition, a culture of MR522::pSJM107, presumably a mixture of both red and yellow colony types, was shown to exhibit constitutive expression of p^{QRS} (data not shown).

These results are consistent with the theory that the carD product acts at a stage prior to the induction of the carQRS region promoter. p^{QRS} is constitutively expressed because of the absence of a functional carR regulator gene and is able to synthesise carotenoids as a result of

this expression. It does not, however, preclude any later role for this product. It could, for example, be involved in augmentation of the function of the carQ product; a role rendered unnecessary by high cellular levels of this product caused by integration of pSJM107.

At present we do not know the nature of the product(s) encoded by carD. The Car$^-$ phenotype is not the only phenotype caused by integration of the transposon at this locus. Upon starvation, cells of strain MR522 are unable to aggregate and undergo sporulation (Nicolas F. and Murillo F., pers. comm.). Because this mutation has such pleiotropic effects, it remains a formal possibility that the carD locus is not directly involved in the functioning of the carQRS operon. Work is now underway in another laboratory to clone and sequence this locus.

5.6 Discussion:

We have described in the "sequestration" model above, how a ratio of 1:1 between the carQ and carR genes is essential for the correct regulation of P^{QRS}. The sequence of the region reveals that the two genes are translationally coupled resulting in translation of their gene products, again, in a precise 1:1 ratio.

If our model is correct, then this would imply that any CarQ protein will always be immediately sequestered by the CarR protein translated from the same mRNA template and by the same ribosome. If these two genes were not translationally coupled, then it is possible that the correct functioning of the light-inducible promoter would be upset. This aspect of the model is addressed in the following chapter.

Figure 5.6 Summary of results of experiments involving an alteration of the ratio of carQ to carR genes: These experiments have either been carried out by other members of the group (nos. 3 and 4) or as part of this study.

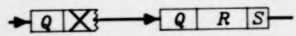
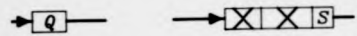
1) MR135::pSJM107 - The light-inducible promoter (P^{QRS}) drives carQ expression at the Mx8 attachment site on the chromosome (Section 5.4.1).

2) DK101::pSJM107 - P^{QRS} drives expression of carQ at the Mx8 attachment site, but this time in a wild-type background. A wild-type carQRS operon, therefore, is present at a different part of the chromosome (Section 5.4.2).

3) The integration by homologous recombination of a fragment of DNA encoding carQ and a truncated form of carR, results in two carQ genes and only one viable carR gene (Section 5.5.2).

4) Integration by homologous recombination of a second carQRS operon encoding an insertionally inactivated carQ gene, results in only one viable carQ gene and two carR genes (Section 5.5.3).

5) MR132::pSJM107 - P^{QRS} drives expression of carQ at the Mx8 attachment site. A carQRS operon is encoded at another part of the chromosome, however, mutations within both the carQ and carR genes in this operon, results in the carQ:carR ratio of the strain being 1:0. In this strain constitutive expression of carQ, results in constitutive expression of the proposed activator of carotenogenesis encoded by carS (Section 5.5.4). Although this ratio is the same as MR135::pSJM107, therefore, the Car phenotype is not Car^{-}, but, Car^{C}.

genotype.	*carQ* : *carR* ratio.	Car phenotype.
1) 	1:0	Car^{-}
2) 	2:1	Car^{c}
3) 	2:1	Car^{c}
4) 	1:2	Car^{+}
5) 	1:0	Car^{c}

The model requires that the promoter in the dark is not completely repressed. If this were to happen then neither the regulator nor the activator of the promoter would be expressed and induction of expression could never take place. Instead, we envisage a steady background activity of the promoter in just the same way that other negatively regulated promoters require such activity (Regulation of the lac operon of E. coli, for example, requires a basal level of lacZYA transcription for the conversion by the lacZ gene product, of lactose to allolactose. The latter is the inducer of the lac operon promoter; Miller and Reznikoff, 1980). Just such a steady background level of activity has been observed in the dark when P^{QRS} is fused to a promoterless lacZ gene (Figure 3.6).

Finally, although the model requires that certain functions are carried out, we do not know (or need to know for the purposes of the model) how these functions are achieved. For example, although we now believe the carQ gene product activates the light-inducible promoter, we do not know how this activation is achieved. As has already been discussed, the derived amino acid sequence of the CarQ protein shows no homology to any known DNA binding proteins or to any sigma factors (McGowan, 1989). Work is underway within the group to characterise the binding of any proteins to the promoter under light-inducing conditions in an effort to address this problem.

Similarly, we do not know how the CarR protein is able to perceive the light signal, or how it then goes on to "release" the CarQ protein. It is possible that when in contact with singlet oxygen produced by the action of light upon protoporphyrin IX, the CarR protein undergoes a conformational change such that it can no longer bind the CarQ protein.

Work within the group using antibodies directed against a carR/lacZ

protein fusion, would seem to support an alternative theory. Using these antibodies, the CarR protein could not be detected after the cells had been placed in the light (Gorham H., pers. comm.). This result was surprising because CarR could be detected in the membranes of dark grown cells. Light-induction of the cells results in a greatly increased expression of *carR* and our inability to detect the protein under these circumstances indicates, perhaps, that it has been destroyed altogether in the light. This destruction of CarR could potentially be carried out by a protease that is activated by light/singlet oxygen.

A likely candidate for the gene encoding such a protease would be *carD*. This would explain why a *carD*$^-$ strain is unable to produce carotenoids in the light: sequestration of the CarQ protein would continue in the absence of CarR protein destruction. The encoding of a protease function might also explain the apparent pleiotropic nature of the *carD*$^-$ mutation.

In this regard, a further point is noted with interest. Such a light-activated proteolytic activity has already been reported (Vass *et al.*, 1992). Damage to one of the reaction centre subunits, D1, of photosystem II during plant photosynthesis, is caused by singlet oxygen. The singlet oxygen is believed to be formed by the action of light upon certain forms of chlorophyll. The damaged D1 protein is then in turn triggered for proteolytic degradation.

The similarities between this system and events prior to light-induced carotenogenesis are striking. Singlet oxygen is believed to be formed in the membrane of *M. xanthus* by the action of light upon protoporphyrin IX. Furthermore the same oxygen species has also been shown to induce carotenogenesis (Robeson, 1992 and described in Section 1.7.1). It is possible to imagine a situation in which singlet oxygen damages a membrane bound

CarR protein, causing the latter to become triggered for degradation by a *carD* encoded protease. Such degradation would lead to the release of the sequestered CarQ protein and hence to activation of carotenogenesis.

Determination of the DNA sequence of *carD* currently underway (Murillo F., pers. comm.), is likely to show whether this gene does indeed encode a protease function. In addition, work is underway within the group to determine whether CarR can be detected in the *carD*$^-$ strain, MR522.

Chapter 6.

carQ/carR Translational Coupling.

6.1 Introduction:

The two genes, carQ and carR are thought to be translationally coupled (McGowan, 1988; Figure 6.1). It has been proposed that the translational coupling of two genes results in their protein products being produced within the cell in equimolar amounts (Oppenheim and Yanofsky, 1980). As described in Chapter 5, this is thought to be of importance in the correct functioning of the carQRS light-inducible promoter. We believe that the "sequestration" model we describe, dictates that the two gene products of carQ and carR must be found within the cell in a one to one ratio. In addition, we would assume that following their translation, the CarQ protein must immediately be sequestered to the membrane by the CarR protein.

...GlyGluLeuGluLeuGluValAlaArgEnd
...GGGGAGCTGGAGCTGGAGGTGGCCCGATGAAGCCACCGATGGACCTGGAC...
MetLysProProMetAspLeuAsp...

Figure 6.1 The junction between carQ and carR: The stop codon of the upstream carQ gene overlaps the start codon of the downstream carR gene. This arrangement is believed to result in the coupling of translation of the two genes (Oppenheim and Yanofsky, 1980).

Translational coupling of these two genes would benefit both of these processes. Under non-inducing conditions, it would prevent more CarQ protein than CarR protein being created. Coupling of the two genes would ensure that whenever a molecule of CarQ is produced, one molecule of the regulator, CarR is translated immediately afterwards. Thus, even though an effective activator in CarQ is produced by background promoter activity, increased expression of the promoter in the dark does not result. In addition, translational coupling could facilitate the immediate sequestration of CarQ by CarR implied by the model. This is discussed further at the end of this chapter.

In order to test this aspect of the model, we set out to uncouple the translation of these two genes. We were able to acheive this using DNA derived from strain MR171 (Chapter 4). The carQRS operon of this strain encodes a deleted and inactive version of carQ (the carQ2 mutation), rendering the strain Car$^-$. The 12bp deletion between positions 1647 and 1659 is in-frame and translation of both downstream genes, carR and carS, therefore, procedes normally.

We wished to complement this mutation in the carQ gene using the plasmid, pSJM107 (Chapter 5). This plasmid is able to integrate into the chromosome at the Mx8 attachment site and encodes both the light-inducible promoter and carQ. Since the viable form of carQ and the carR gene would be expressed from separate versions of P^{QRS}, uncoupling of their translation would be achieved.

Strain MR171 still encodes the parental carR4 mutation of strain DK408 in the carR gene at position 2354. The first stage in this experiment, therefore, was the creation of a strain derived from strain MR171 that contained the 12bp carQ2 deletion but not the carR4 point mutation.

The construction of this carQ⁻ strain of M. xanthus was carried out by first cloning a fragment of the chromosome of strain MR171 encoding the carQ2 deletion. The resulting plasmid was then used to transduce the wild-type M. xanthus strain, DK101. Finally, a strain was isolated in which the chromosome had undergone a homologous recombination event in which the plasmid DNA was deleted. This "looping out" of the plasmid resulted in replacement of the wild-type carQ gene with its deleted allele.

6.2 Cloning of carQ2 mutation:

6.2.1 Creation of strain DK101::pSJM122:

The plasmid pSJM112 (Chapter 4) encoding DNA derived from MR171, was digested with SacI (restricting the insert at positions 865 and 2232) and a 1355bp fragment was isolated by gel electrophoresis. This fragment, encoding the light-inducible promoter, the carQ2 allele and a truncated carR gene, was cloned into plasmid pDAH218 (Hodgson D., pers. comm.) and named pSJM122.

The plasmid pDAH218 encodes the incompatibility region of P1 and a kanamycin resistance gene and can, therefore, be packaged by P1 and used to transduce M. xanthus. It also encodes the cer site of ColE1 (Summers and Sherratt, 1984). This small region of DNA reduces the number of plasmid multimers formed, thus making it more likely that each cell of M. xanthus is transduced by only a single copy of the plasmid.

The plasmid pSJM122 was used to transduce using P1, the M. xanthus wild-type strain DK101. Several colony types were observed following this

The construction of this carO⁻ strain of M. xanthus was carried out by first cloning a fragment of the chromosome of strain MR171 encoding the carO2 deletion. The resulting plasmid was then used to transduce the wild-type M. xanthus strain, DK101. Finally, a strain was isolated in which the chromosome had undergone a homologous recombination event in which the plasmid DNA was deleted. This "looping out" of the plasmid resulted in replacement of the wild-type carO gene with its deleted allele.

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The plasmid pSJM122 was used to transduce using P1, the M. xanthus wild-type strain DK101. Several colony types were observed following this

transduction and are described in Table 6.1. Two of these colony types are shown in Figure 6.2.

Table 6.1 Relative proportions of colony types following transduction of strain DK101 with pSJM122: The figures presented are the combined results of two experiments.

colony type	proportion
red, Car^C	10.8% (+/-1.5%)
yellow, Car^-	6.3% (+/-0.5%)
yellow, Car^+	55.7% (+/-5%)
slightly orange (Car^C)	27.4% (+/-4%)

6.2.2 Analysis of colony types:

We have assumed that the two minor classes of colony types are the result of gene conversion between the carQRS operon on the chromosome and the incoming DNA fragment during homologous recombination. The incoming DNA encodes P^{QRS}, carQ and a truncated form of carR. If the incoming $carQ^-$ gene is gene converted to the wild-type form, this would result in two $carQ^+$ genes and only one functional carR gene on the chromosome. In such a situation, we have already demonstrated P^{QRS} to be permanently activated (Section 5.5.2). Gene conversion of the transducing DNA would, therefore, result in the observed red, Car^C phenotype.

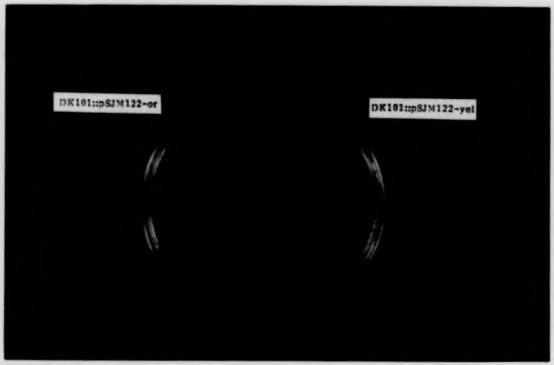


Figure 6.2 Strains derived from the two major colony types following transduction of the M. xanthus strain DK101 with plasmid pSJM122: A strain derived from one of the "slightly orange", Car^C colonies has been named, DK101::pSJM122-or. Similarly, a strain derived from one of the yellow, Car^+ colonies has been named, DK101::pSJM122-yel.

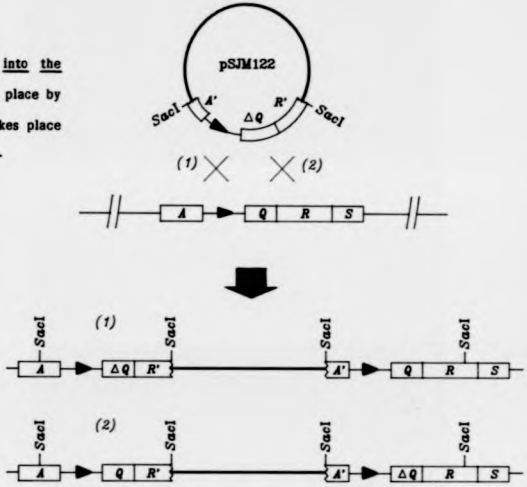
Similarly, if the carQ gene at the carQRS operon on the chromosome becomes gene converted to the incoming carQ2 allele, a wild-type form of the gene will no longer be present. Obviously, with no CarQ activator, the promoter will remain inactive, resulting in a Car^- phenotype. Relatively high frequencies of gene conversion during similar experiments have been observed before, both within the group and elsewhere (Kaiser, 1991).

During integration of plasmid pSJM122 at its homologue in the carQRS operon, two genotypes may be formed depending upon the position of the crossover (Figure 6.3). If the crossover takes place to one side of the 12bp deletion on the plasmid (position 1 in Figure 6.3), a wild-type carQRS operon is reformed. On the other side of the integrated plasmid DNA, a second copy of the promoter drives the expression of a deleted form of carQ and a truncated form of carR.

If the crossover takes place on the other side of the deletion, however, (position 2) a wild-type carQRS operon is not formed. In this case, the wild-type carQ gene is driven by one copy of P^{QRS}, whilst the wild-type carR gene is driven by a second copy of the promoter. Each wild-type gene is coupled to a defective version of the other. In this particular strain, therefore, translation of the viable forms of the carQ and carR genes has become uncoupled.

We believe that this provides us with an explanation for the two major classes of colony types observed following integration of this plasmid. We would predict that the yellow, Car^+ phenotype is displayed by those cells that have undergone recombination via position 1 of Figure 6.3 to reform a wild-type carQRS operon. In this strain, the carQ and carR genes remain translationally coupled and, therefore, P^{QRS} remains correctly regulated.

Figure 6.3 Integration of plasmid pSJM122 into the chromosome of strain DK101: Integration takes place by homologous recombination. If the crossover takes place at position 1, a wild-type carQRS operon is re-formed. On the other side of the integrated plasmid DNA, a second copy of p^{QRS} drives expression of a deleted form of carQ and a truncated carR. If the crossover takes place on the other side of the site of deletion at position 2, each wild-type gene is coupled to a defective version of the other. In this strain, translation of the viable forms of carQ and carR has become uncoupled.



Similarly, we would expect the second, slightly orange phenotype to be exhibited after recombination via position 2 of Figure 6.3.

This interpretation of the results is supported to a large extent, by the relative frequencies of the two colony types. The site of the 12bp deletion is not directly in the centre of the cloned fragment in plasmid pSJM122. A greater number of crossover events were, therefore, expected to occur upstream of this site, where there is a larger region of homology with the chromosome. This greater frequency would give rise to the larger numbers of yellow colonies than orange colonies observed (Table 6.1).

Although experiments designed to confirm the genotypes leading to both colony types have been unsuccessful, we still assume that the above described explanation for their formation is correct. Work described below relies to a large degree upon this assumption.

It had already been predicted using the sequestration model that uncoupling the translation of these two genes could lead to the incorrect regulation of P^{QRS} activity (Sections 5.8 and 6.1). Since the two genes are no longer translationally coupled in strain DK101::pSJM122-or, some of the CarQ protein produced by the background level of promoter activity, is now available to further activate the promoter and thus cause carotenogenesis. That this activation must involve only a proportion of the CarQ protein produced, is evident from the "slightly orange" phenotype of these colonies. The apparent amount of carotenoids produced (and, therefore, the level of activity of P^{QRS}) is far less than the normal light-induced production of carotenoids. We would conclude, therefore, that the CarR protein is still able to sequester some of the CarQ protein.

6.3 Generation of UWM303 - a carQ⁻ strain of M. xanthus:

6.3.1 Further experimental aims:

Although we had already succeded in uncoupling the translation of carQ and carR, we decided for a number of reasons to continue with the experiment as described in Section 6.1. This involved the construction of the originally proposed strain containing the carQ2 mutation in a wild-type background. This mutation could then be complemented with the wild-type allele inserted at the Mx8 attachment site.

Because all the resulting colonies would contain untranslationally coupled carQ and carR genes, the results of such an experiment would require less interpretation. Secondly, although we had already observed orange colonies resulting from the uncoupled translation, we were not able to measure the evident activity of P^{QRS} under these circumstances. Finally, a carQ⁻ strain without the parental mutation in the carR gene could be used in other investigations into aspects of the carQRS operon (Chapter 7).

6.3.2 Spontaneous recombination:

We have described above how the chromosome of DK101::pSJM122-or contains a duplication of part of the carQRS operon on either side of the integrated vector. If homologous recombination between these direct repeats takes place, the plasmid is "looped out" of the chromosome and lost (Figure 6.4). We decided to isolate a strain that had undergone such a recombination event, to replace the wild-type carQ gene with the carQ2 mutation of MR171.

During the recombination event, the crossover between the two direct repeats could take place on either side of the carO2 mutation. If the crossover took place upstream of the mutation (position 1 of Figure 6.4), then the plasmid DNA that was looped out of the chromosome would encode the wild-type carO allele. This event would leave a carQRS operon on the chromosome that included the carO2 mutation and the resulting strain would be Car$^-$. If the crossover took place on the other side of the mutation (position 2), the plasmid pSJM122 would be regenerated and the carQRS operon remaining on the chromosome would be wild-type. Only some of the cells resulting from this spontaneous recombination event, therefore, would exhibit the required carO$^-$ genotype.

Spontaneous recombination between the two direct repeats cannot be selected for and cannot be induced. In order to provide an opportunity for this event to occur, therefore, we continually sub-cultured a 10ml culture of DK101::pSJM122-or. 0.01ml of a two day old culture were used to inoculate 10ml of DCY and the resulting sub-culture itself incubated for a further two days.

This process was repeated for 13 successive sub-cultures before the cells were diluted and subsequently grown on DCY plates. Five hundred and fifty of the resulting colonies were replicated onto both DCY plates and DCY + kanamycin plates. Five of these colonies were found to be kanamycin sensitive. Of these five, two were subsequently found to be Car$^-$ and three Car$^+$. The two Car$^-$ colonies were assumed to have the carO$^-$ genotype expected and the strains derived from these colonies were named, UWM303 and UWM304.

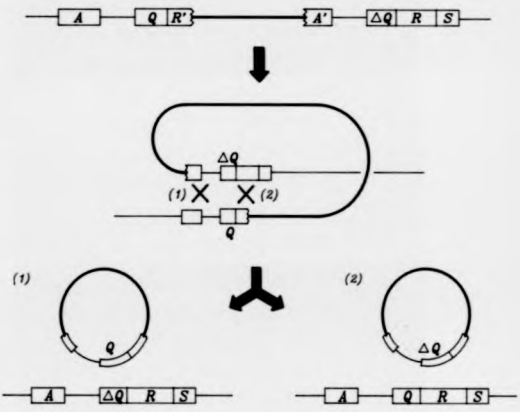


Figure 6.4 Spontaneous homologous recombination between the direct repeats of strain DK101::pSJM122-or: The position of the crossover during this recombination dictates the genotype of the resulting strain. A crossover at position 1 will lead to the formation of a carQRS operon that encodes the carQ2 allele. If the crossover takes place at position 2, the original plasmid, pSJM122 is looped out to leave a wild-type, chromosomally encoded carQRS operon.

6.3.3 P^{QRS} activity in the *carQ*$^-$ strain, UWM303:

The level of activity of P^{QRS} in the *carQ*$^-$ strain UWM303 was assayed using the plasmid, pDAH217 (Hodgson D., pers. comm.). This plasmid has been constructed such that part of the *carQRS* operon encoding the light-inducible promoter drives expression of a promoterless *lacZ* gene. When *M. xanthus* is transduced using P1 with this plasmid, it is able to integrate into the chromosome by homologous recombination at the *carQRS* operon. The level of activity of the native P^{QRS} may then be assayed by the level of expression of the *lacZ* gene. Following transduction of UWM303 with pDAH217, the activity of the promoter was assayed in both the light and the dark.

The results of this experiment (Figure 6.5) show that there is no expression of P^{QRS} in the absence of a wild-type *carQ* gene. This result is expected and indicates that the Car$^-$ phenotype of UWM303 is caused by the non-expression of the promoter in the light and not by a second mutation within a gene involved later in the *car* regulon. It also confirms some of the conclusions reached in Chapter 5, where we stated that *carQ* expression is required for expression of the light-inducible promoter.

Although no increased expression in response to light is observed, the actual level of activity of P^{QRS} in both the light and the dark is higher than normal background activity. The wild-type level of expression of the promoter in the dark is around 5 units (Figure 3.6), whereas the level of activity in the presence of the *carQ2* mutation in strain UWM303::pDAH217 is around three times this level. There is no obvious reason for this increase in background expression.

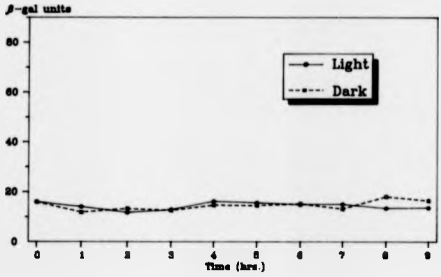


Figure 6.5 β-galactosidase activity of strain UWM303::pDAH217: Strain UWM303, a strain in which a small region of the carQ gene had been deleted, was transduced with pDAH217. This plasmid encodes P^{QRS} which drives expression of lacZ and integrates by homologous recombination at the wild-type carQRS operon.

6.3.4 UWM303::pSJM107:

In order to carry out the originally proposed experiment involving complementation of the carQ2 mutation with the wild-type allele, strain UWM303 was transduced using P1 with pSJM107 (Section 5.2). This plasmid encodes P^{QRS} driving expression of carQ and does not encode either carR or carS. It can integrate into the chromosome of M. xanthus at the Mx8 attachment site, attB, by site specific recombination.

The majority of the resulting transduced colonies were Car^{C}. This phenotype was an intermediate between the slightly orange phenotype of strain DK101::pSJM122-or (where carQ and carR are no longer translationally coupled) and that produced when the promoter is unregulated in the presence of an extra copy of carQ (in strain DK101::pSJM107; Figure 6.6). The minority of colonies with differing phenotypes were believed to result from integration of the plasmid by homologous recombination at the carQRS region and/or gene conversion of one of the carQ alleles. These colonies were, therefore, ignored.

The level of β-galactosidase produced by strain UWM303::pSJM107 was assayed in both the light and the dark (Figure 6.7). The results show that the integrated P^{QRS} exhibits constitutive expression of around 700 units. This level of activity is around half the level of activity produced by the unregulated promoter (Figure 5.4 - P^{QRS} in the presence of an extra carQ gene)

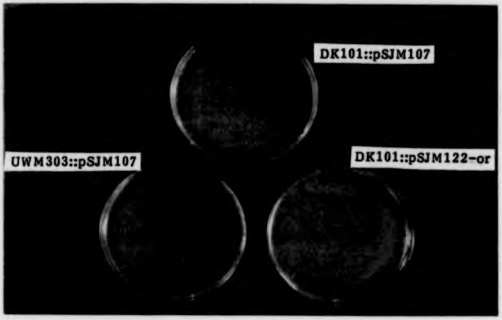


Figure 6.6 Comparison between the Car^C phenotypes of UWM303::pSJM107, DK101::pSJM107 and DK101::pSJM122-or: The Car^C phenotype of strain DK101::pSJM107 is a result of the introduction into the chromosome of an extra copy of the P^{QBS} activating gene, carQ (Chapter 5). In contrast, the Car^C phenotypes of strains UWM303::pSJM107 and DK101::pSJM122-or result from the uncoupling of translation of carQ and carR. In the chromosome of strain DK101::pSJM122-or, these two genes are separated only by the length of the inserted vector DNA. In strain UWM303::pSJM107, however, the two genes are separated by a large part of the chromosome.

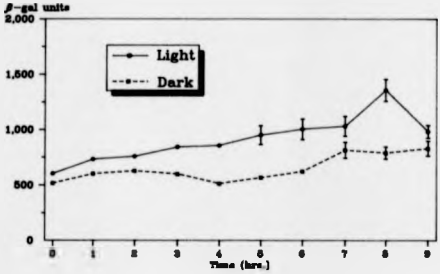


Figure 6.7 β-galactosidase activity of strain UWM303::pSJM107: Strain UWM303, a strain in which a small region of the carQ gene has been deleted, was transduced with pSJM107. This plasmid encodes P^{QRS} which drives expression of carQ and lacZ and integrates by site specific recombination at the Mx8 attB site on the chromosome.

8.4 Uncoupling of translation or carO⁻/carO⁺ competition?

8.4.1 Integration of the carO2 allele at attB:

Using the sequestration model described in Chapter 5, it is possible to construct an alternative explanation for the Car^C phenotypes of strains DK101::pSJM122-or and UWM303::pSJM107. It is possible that the CarQ protein produced by the carO2 allelle, competes with the wild-type form for binding to the CarR protein. If this were to happen, some of the wild-type form would become available within the cell for the activation of p^{QRS}. In order to test this theory, we decided to create a strain in which the deleted form of the carO gene had been inserted into the chromosome of strain DK101, at the Mx8 attachment site. This experiment would result in the re-establishment of translational coupling of the wild-type carQ and carR genes, but in the presence of the carO2 allele.

Construction of this strain was carried out in several stages. Firstly, the 1355bp SacI/SacI fragment of pSJM112 (Chapter 4) was cloned into the SacI digested plasmid pIC19R. The resulting plasmid was named pSJM134. Secondly, the insert of pSJM134 was cut out at the two flanking EcoRI sites and ligated into the plasmid pSJM103 at the unique EcoRI site. Finally, strain DK101 was transduced using the bacteriophage P1, with this plasmid, named pSJM136.

Apart from a minority class of red, Car^C colonies, again assumed to be the result of gene conversion, all colonies were yellow. Upon further testing of 80 of these yellow colonies, all were found to produce carotenoids upon exposure to light. In addition, these colonies were also found to exhibit a previously unknown phenotype, Car^{ts}. At 33°C in the dark, the

cells remained yellow. In the dark at 18°C, however, the cells became orange with the accumulation of carotenoids (Figure 6.8). Because the integrated *carQ* gene of pSJM136 is fused to a promoterless *lacZ* gene within the chromosome of this strain, we were able to assay the level of P^{QRS} activity caused by this temperature shift. When the levels of β-galactosidase were assayed at 33°C and 18°C, the latter temperature was found to cause a rise of around ten fold in expression of the promoter above that at 33°C (Figure 6.8).

6.4.2 Integration of the *carQ1* allele at *attB*:

When P^{QRS} drives expression of the *carQ2* allele at the Mx8 attachment site, *attB*, a Car^{ts} phenotype results. Upon further examination of the two strains DK101::pSJM122-or and DK101::pSJM122-yel, we observed a similar increase in carotenoid production at 18°C compared to that at 33°C (data not shown). We wished to determine, therefore, whether another deletion mutation within *carQ*, the *carQ1* mutation of strain MB132, could produce the same phenotype.

In order to carry out this experiment, we cloned the 1358bp *Sac*I/*Sac*I fragment of pSJM110 (Chapter 4) into the *Sac*I digested plasmid pIC19R. The resulting plasmid was named pSJM145. This insert of this plasmid was subsequently re-isolated by restriction at the two flanking *Eco*RI sites and ligated into the plasmid pSJM103 at the unique *Eco*RI site. This plasmid, named pSJM146, was used to transduce using P1, strain DK101.

Examination of the resulting strain, DK101::pSJM146, showed that insertion of the *carQ1* allele into the chromosome at the Mx8 *attB* site did not result in a Car^{ts} phenotype.

DK101 (wild-type) DK101::pSJM136

Figure 6.8 Car^{ts} phenotype of strain DK101::pSJM136: The presence of the carQ2 allele at the Mx8 attB site causes carotenoid production when the cells are incubated at 18°C in the dark (shown above), but not at 33°C in the dark. This phenotype may be compared with that of the wild-type strain DK101.

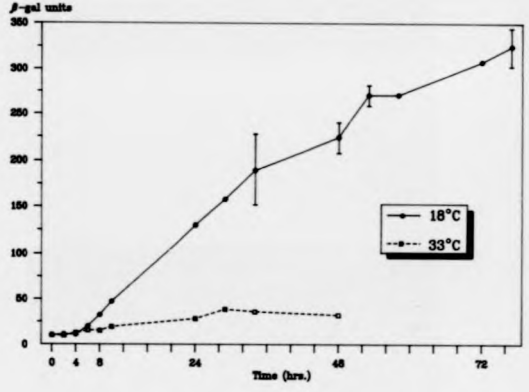


Figure 6.9 β-galactosidase activity of strain DK101::pSJM136: The wild-type strain, DK101, was transduced with the plasmid, pSJM136. This plasmid encodes P^{QRS} which drives expression of the carQ2 allele and lacZ, and integrates into the chromosome at the Mx8 attB site.

This was confirmed by an analysis of the levels of β-galactosidase produced by P^{QRS} under these conditions (Figure 6.10). Although in the dark the level of activity of the promoter appeared to be slightly above that in a wild-type background, there was no difference in activity between that at 33°C and at 18°C. We conclude from this experiment that the Car^{ts} phenotype observed is allele specific and is restricted to the carQ2 allele alone.

6.5 Discussion:

6.5.1 Uncoupling of translation of carQ and carR, results in a Car^{c} phenotype:

In the Car^{-} strains MR171 and UWM303, the carQ2 mutation renders the carQ gene unable to activate expression of P^{QRS} at any temperature (data not shown). The carQ2 allele cannot, therefore, cause temperature sensitive promoter activation. The observed expression of the promoter in strain DK101::pSJM136 in the dark at 18°C must be due to the CarQ protein produced by the wild-type carQ allele.

In order to explain the temperature sensitive nature of this expression in terms of the sequestration model, we assume that the mutated CarQ protein can bind to CarR only at the lower temperature. At 18°C, therefore, it competes for binding to CarR with the wild-type form, thus freeing some of the latter to activate promoter expression. At 33°C, we assume that the mutated CarQ protein can no longer bind to CarR, possibly due to a conformational change. At this temperature, there is no competition to upset the 1:1 ratio of CarQ to CarR and the cells remain yellow.

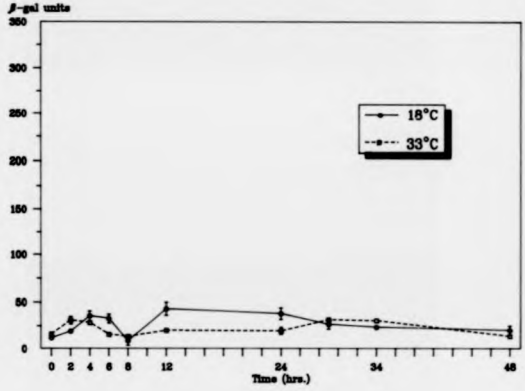


Figure 6.10 β-galactosidase activity of strain DK101::pSJM146: The wild-type strain, DK101, was transduced with the plasmid, pSJM146. This plasmid encodes P^{QRS} which drives expression of the carQ1 allele and lacZ, and integrates into the chromosome at the Mx8 attB site.

We have shown in the above described experiments, that competition between the two forms of CarQ protein does take place. This competition, however, only occurs at 18°C and as we have shown, is allele specific. Any change in the Car phenotype at temperatures above 18°C must be due to another factor. The strains DK101::pSJM122-or and UWM303::pSJM107, are orange at both 33°C and 18°C. We conclude, therefore, that these Car^C phenotypes are due to the uncoupling of translation of the carQ and carR genes.

6.5.2 Translational coupling and the regulation of expression of P^{QRS}:

As discussed in the introduction to this chapter, uncoupling the translation of carQ and carR could lead to a breakdown in the regulation of the light-inducible promoter in two ways. If expression of the two genes occurs at different rates in the absence of coupling, then more CarQ than CarR could be produced. In the dark, this would lead an increase in the expression of the carQRS operon and hence to a Car^C phenotype.

Alternatively, if translation of the two genes occurs at different positions on the chromosome, immediate sequestration of CarQ by CarR would be unlikely. Again, because of the increase in the cellular concentration of free CarQ activator, this would lead to a Car^C phenotype. The results of the experiments described do not enable us to determine which of these two possible mechanisms operates to cause the Car^C phenotypes observed.

The Car^C phenotype of UWM303::pSJM107 is more intense than that of DK101::pSJM122-or (Figure 6.6). This difference, however, may be attributed to either of the two mechanisms. In the former strain, expression of

the wild-type carQ gene in the dark might be greater due to the apparently greater background expression of genes inserted at the attB region (Chapter 3). Alternatively, the attB region is some distance from the carQRS operon on the chromosome (Figure 6.11; Chen et al., 1991). The two genes are, therefore, separated by a larger distance in strain UWM303::pSJM107 than in DK101::pSJM122-or, where they are separated only by the inserted DNA of the vector. Thus, sequestration of CarQ by CarR is more likely in the latter strain rather than the former.

6.5.3 Translational coupling facilitates the sequestration of CarQ by CarR:

Translational coupling of carQ and carR could facilitate the sequestration of a CarQ protein by a CarR protein in the model proposed in Chapter 5. During the expression of two translationally coupled genes, translation of the downstream gene is thought to be performed by the ribosome that has just completed translation of the upstream gene (Oppenheim and Yanofsky, 1980). Indeed, the arrangement of the start codon of carR and the stop codon of carQ, where they overlap and the former is in the -1 position with respect to the latter, has been shown to be optimal in causing this ribosomal re-initiation (Figure 6.1; van de Guchte et al., 1991).

As described in Figure 6.12, we have developed the sequestration model to accomodate the coupling of translation of the carQ and carR genes. We envisage a situation in which a newly formed CarQ immediately binds to the amino terminal end of the CarR protein that is translated by the same ribosome. This could occur, as shown in Figure 6.12, even before the latter has been fully translated.

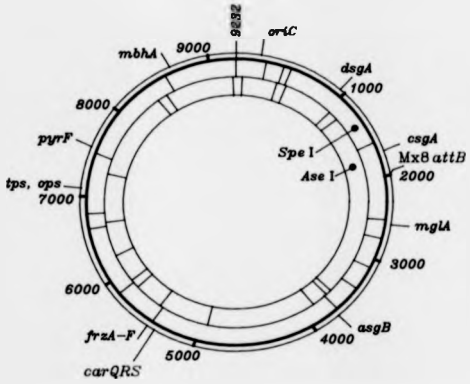


Figure 6.11 Physical map of the chromosome of M. xanthus: (adapted from Chen et al., 1991). The carQRS operon and the Mx8 attachment site, attB, are separated by a large part of the chromosome.

Upon completion of translation of the CarR protein, it is folded into the membrane, but has bound to its amino terminal end, a CarQ protein. This model not only describes a method by which sequestration may be set up, but also ensures that no free CarQ protein is available to further activate P^{QRS}.

We have no evidence for the role of the amino terminal end of CarR, or indeed any portion of this protein, in the binding of CarQ called for in the sequestration model. We would point out, however, that unlike the majority of this protein, the hydrophilic nature of the amino terminal end means it is unlikely to be associated with the membrane and, therefore, is available for such a role.

8.5.4 Translational coupling of carR and carS:

The stop codon of carR and the start codon of carS also overlap and again the two genes are believed to be translationally coupled (McGowan, 1989). As we will show later in this study, however, the carS gene product is not involved in either the functioning or regulation of the light-inducible promoter. The apparent translational coupling of carR and carS, therefore, is probably not as significant as that between carQ and carR. Instead, it may simply be a means to ensure the highly efficient translation of carS.

We will also show later in this study, that carQ, like carS, encodes an activator of some of the carotenoid biosynthetic genes. The effective translational coupling of carQ with carS (via carR) means that the two activators encoded by these genes would be present in the cell in equimolar amounts. Translational coupling has been reported elsewhere to control relative amounts of enzymes in a biosynthetic pathway (galT and

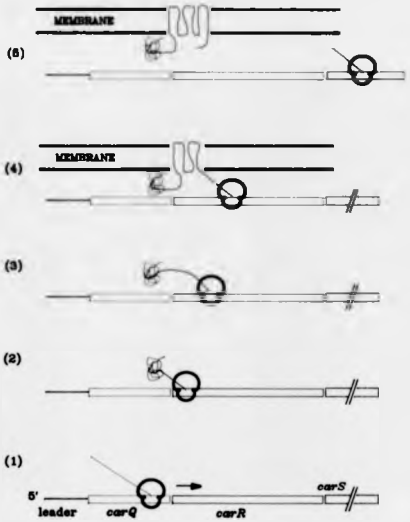


Figure 6.12 Translation of carQ and carR: Translational coupling of carQ and carR could facilitate the immediate sequestration of CarQ by CarR in the model proposed in Chapter 5. A newly formed CarQ could potentially bind to the amino terminal end of the CarR protein that is subsequently translated by the same ribosome (2). By its hydrophilic nature, this part of CarR would not appear to be membrane associated. Once fully translated, CarR would be located in the membrane (5). In this way, each CarQ protein produced by background promoter activity in the dark would be sequestered to the membrane and could not further activate the promoter.

galK of E. coli, for example, are translationally coupled genes encoding enzymes required during galactose metabolism; Schumperli et al., 1982).

It is interesting to note in this regard that there is only very poor homology with the 3' end of the Myxococcus xanthus 16S ribosomal RNA (Oyaizu and Woese, 1985) upstream of the carS start codon (McGowan, 1989). This is in contrast to the upstream regions of carQ, carR and gufA, where such homology does exist. This homology is believed to play a role in the initiation of translation and, in its absence, translation of carS may have to rely solely upon coupling with that of carR.

As described in Chapter 1, carS is believed to encode an activator of the car regulon. The efficient translation of carS, resulting in the rapid accumulation of a large amount of the activator, would ensure a swift response to light by the carotenoid synthesising genes. We would predict, therefore, that any experiment designed to upset the translational coupling of carR and carS, could result in a decrease in the intensity of the carotenogenic response if not its prevention. If such an observation were made, we would further predict that insertion of a ribosome binding site at an appropriate distance upstream of the uncoupled carS start codon, would restore a wild-type Car phenotype.

Chapter 7.

carQRS Encoded Activation of Other car Loci.

7.1. Introduction:

We have already described evidence that seems to implicate the carS gene product in the carQRS encoded activation of the genes at carB and carC (Sections 4.3.3 and 5.4.2). This was further supported by the results of a series of experiments carried out within the group (Hodgson, in press and outlined in Section 1.7.4).

Evidence has been reported elsewhere, however, that seems to conflict with this hypothesis. Using the transposon Tn*5*ΩDK1810 (Martinez-Laborda *et al*., 1986) to constitutively drive expression of the carQRS operon, activation of carB and carC was assayed. Whereas carB was shown to be constitutively expressed (Balsalobre *et al*., 1987), no expression of carC was observed (Fontes and Murillo, 1986). The different patterns of expression between carB and carC would seem to show that these two loci are responding to a different activator. Possibly, the genes at carB are activated by the carS gene product and those at carC are activated by a second, unidentified protein.

In order to test whether the carS gene product does indeed activate either carB or carC, it was decided to create a strain in which the carS gene had been deleted. We wished to then insert into the chromosome of this strain, a series of promoter probes to assay the activity of car-related promoters in the carS$^-$ background. To this end, the strain had to be constructed to remain kanamycin sensitive. Resistance to kanamycin could then be used to select for integration of these promoter probes.

7.2 Construction of a carS⁻ strain:

7.2.1 Construction of plasmid pSJM140:

In order to create a carS⁻ strain, a number of *in vitro* plasmid manipulations were carried out (Figure 7.1). The first step involved the sub-cloning of a fragment of the *M. xanthus* chromosome into a plasmid. We ligated the 1743bp fragment of pDAH194 (Hodgson D., pers. comm.), from the MluI site at position 1391 to the XhoI site at position 3134, into plasmid pMTL21 (Chambers *et al.*, 1988) restricted with the same enzymes. This fragment encodes part of carQ, and all of both carR and carS and the resulting plasmid was called pSJM130.

We wished to digest the plasmid pSJM130 with the enzyme, AvaII. This enzyme is blocked by overlapping dcm methylation and, therefore, we isolated the plasmid from the dcm⁻ strain of *E. coli*. GM48. There are two AvaII sites within the carS gene at positions 2359 and 2649, such that digestion followed by religation would delete part of the coding sequence of this gene (Figure 7.2).

The plasmid pSJM130 has eight AvaII sites, however, and in order to cut only those sites within the carS gene, partial digestions using decreasing dilutions of the enzyme AvaII were carried out. Following gel electrophoresis of the partially digested DNA, fragments of approximately the correct size were isolated, religated and used to transform *E. coli* strain GM48. Plasmids from the resulting transformed colonies were isolated and restricted with AvaII. One of these plasmids was shown to no longer contain the 290bp fragment encoding part of carS and was named pSJM132. This deletion within the carS gene has been named the carS2 mutation.

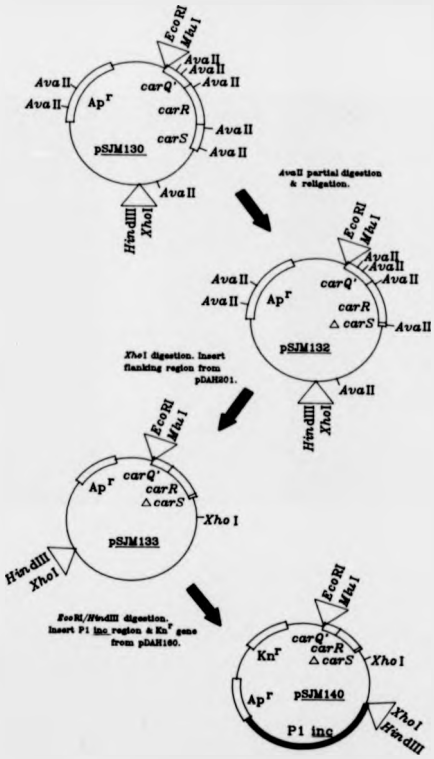


Figure 7.1 Construction of plasmid pSJM140:

```
                                2340        2350        2360        2370        2380
                    ..... AAACCCCGTT CCTACCCCCC ATGATCCAGG ACCCCTCACT CATCATCTCC
                                                MetIleGlnA spProSerLe ulIleIleCys

      2390        2400        2410        2420        2430        2440        2450
CATCACGTGG ACCCCCCACC CGTCCCCATT CCCCCGAACG TGAAGGTCGT TCCCCACTCC GACGACCCCA
HisAspValA spGlyAlaPr oValArgIle GlyAlaLysV alLysValVa lProHisSer GluAspGlyT

      2460        2470        2480        2490        2500        2510        2520
CCATCACCCA CCCCTTCCTG CCCCACACCC GAATCGTCGT CCCCCTCGTG TTCCACCACC CCCCCACCCA
hrIleSerGl nArgPheLeu GlyGlnThrG lyIleValVa lGlyLeuVal PheAspAspP roAlaThrGl

      2530        2540        2550        2560        2570        2580        2590
GTACCCCCAT GACCCCCTCA TCCACGTCCT CGTCCAACCG CTCCCCCAAG ACCTCTTCTT CCCCCACCAG
nTyrProAsp AspProLeul leGlnValLe uValGluGly LeuGlyGluA spLeuPhePh eProGluGlu

      2600        2610        2620        2630        2640        2650        2660
CTCCAGCTCG CCCCCCAGTG CCCCCCCAAC CCCATTCCCC AGCACCCTCA CCCCTTCCCG ACCCCCCCCC
LeuGluLeuA laProGluTr pAlaArgAsn ArgIleAlaG lnHisArgGl nAlaValArg ThrGlyGlyA

      2670        2680        2690        2700        2710
CGAGTTCCCT GGACCCCCTG CCCTGACGTG GCCCCCCCCG CGTCAGCCCG .....
rgSerSerLe uGluArgLeu ProEnd
```

```
                                2340        2350        2360        2370        2380
                    ..... AAACCCCGTT CCTACCCCCC ATGATCCAGG ACCCCCCCCC CGAGTTCCCT
                                                MetIleGlnA spArgArgPr oGluPheAla

      2390        2400        2410        2420        2430        2440        2450
GGAGCCCCTG CCCTGACGTG GCCCCCCCCG CGTCAGCCCC TACCCCACTG CCCTCCCCCG AACCCCACCG
GlyAlaProA laLeuThrTr pArgAlaPro ArgGlnArgV alProAspCy sAlaArgAla AsnAlaThrA

      2460        2470        2480
CCTGAGAAAT CCCCTTGGAG CTCCGAAAGA .....
laEND
```

Figure 7.2 Comparison between wild-type and deleted versions of carS: The derived amino acid sequence of carS (upper sequence) is compared with that of the same gene following deletion of part of the coding region (lower sequence). DNA was deleted between the two AvaII sites at positions 2359 and 2648 (underlined). Only the first four amino acids of the wild-type CarS protein are translated in the deleted version.

In order to construct the carS⁻ strain, we wished to integrate this plasmid by homologous recombination into the chromosome of M. xanthus. At a later stage, we hoped to be able to select a strain in which this plasmid DNA had "looped out" of the chromosome, again by homologous recombination, to leave behind the carS⁻ mutation.

Although large enough for the initial integration, the remaining insert of pSJM132 was judged to be too small to undergo efficiently this second recombination. We inserted, therefore, the flanking 2.5kb, XhoI/XhoI fragment of pDAH201 (Hodgson D., pers. comm.) into the unique XhoI site of pSJM132. In the wild-type M. xanthus chromosome, this fragment flanks the carQRS operon in this position. The resulting plasmid was named pSJM133.

Finally, in order to transduce M. xanthus using P1 and select for integration of the plasmid, we ligated the insert of pSJM133 into a plasmid that encoded both the incompatibility region of P1 (P1 inc) and a kanamycin resistance gene. This was achieved by digestion of pSJM133 with both EcoRI and HindIII and cloning the resulting 4kb fragment into the vector pDAH180 (Hodgson D., pers. comm.) digested with the same two enzymes. The resulting plasmid was named pSJM140.

7.2.2 Construction of M. xanthus strain UWM301:

M. xanthus strain DK101 was transduced using P1 with pSJM140. Some of the resulting kanamycin resistant colonies were tested for their ability to produce carotenoids in response to light. Since the position of the crossover during homologous recombination could vary, two distinct phenotypes were expected (Figure 7.3).

Those strains in which a crossover took place between the carO gene and the site of deletion were expected to be Car$^-$. This is because P^{QRS} now drives a carQRS operon that does not contain a functional carS gene. Similarly, because a wild-type carQRS operon is regenerated, those strains in which crossovers took place on the other side of the point of deletion were expected to remain Car$^+$.

Furthermore, the distance between the end of the insert in carO and the point of deletion, is smaller than that between the point of deletion and the other end of the insert. A crossover during integration, therefore, was more likely to occur in this larger region of homology and thus more Car$^+$ than Car$^-$ colonies were expected.

Of 36 DK101::pSJM140 colonies tested, 35 remained Car$^+$ and only one had become Car$^-$. This latter, Car$^-$ strain was named UWM300.

The final stage in the construction of the carS$^-$ strain involved a spontaneous recombination event, in which the integrated plasmid in strain UWM300 was "looped out" of the chromosome. Such recombination could take place between the two regions of homology on either side of the integrated plasmid. Once again, the position of the crossover dictated the genotype of the resulting strain (Figure 7.4).

We hoped to be able to isolate a strain with one of the two possible genotypes, in which the wild-type carS gene was looped out of the chromosome and replaced by the deleted version of pSJM140 (option 2 in Figure 7.4). Again, because most crossovers during recombination would take place between the larger regions of homology, we expected that most looping out events would result in the required genotype.

Figure 7.3 Integration of pSJM140 into the chromosome of M. xanthus strain DK101: Plasmid pSJM140 encodes part of carQ, the whole of carR and a deleted form of carS. Integration of this plasmid takes place by homologous recombination. The position of the crossover with respect to the carS gene during integration, dictates the genotype of the resulting strain. A crossover at position 1, upstream of carS, will result in a Car^- strain. This is because P^{QRS} now drives a carQRS operon that does not contain a functional carS gene. Alternatively, the resulting strain will remain Car^+ following a crossover at position 2.

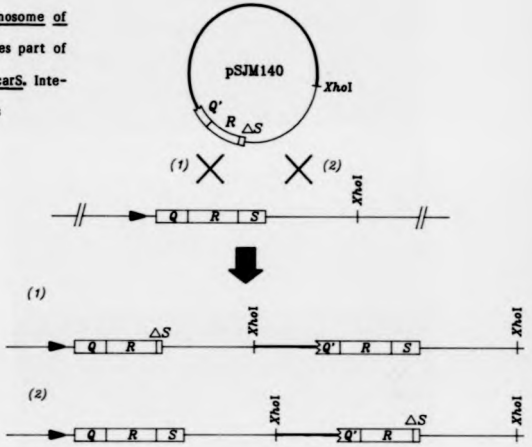
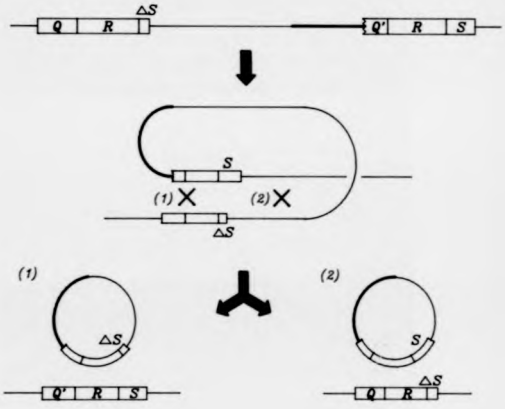


Figure 7.4 Homologous recombination in the chromosome of strain UWM300: The integrated plasmid DNA in UWM300 is flanked by regions of homology. Recombination between these two regions results in "looping out" of the plasmid. The position of the crossover event relative to the two copies of carS, determines the genotype of the resulting strain. A crossover at position 2 will loop out a plasmid encoding a wild-type carS gene, and will leave on the chromosome, a carQRS operon encoding the deleted version of carS.



This recombination event could be neither selected for nor induced. A 10ml culture of strain UWM300 was allowed to grow for two days. 0.01ml of this culture was then used to inoculate another 10ml DCY broth and again allowed to grow for two days. Repetition of this process ensured the growth of many generations of this strain and hence provided the opportunity for the spontaneous recombination to occur.

After nine such sub-cultures, the culture was diluted and subsequently grown on DCY plates. One hundred of the resulting colonies were replicated onto both DCY plates and DCY + kanamycin plates. Two of these colonies were found to be kanamycin sensitive and were tested for their ability to produce carotenoids in response to light. Both were found to be Car$^-$ and it was believed that these two strains were the required carS$^-$ mutants. These two strains were named UWM301 and UWM302.

In order to confirm the genotype of these two strains, Southern blots of chromosomal DNA from strains UWM300, UWM301, UWM302 and DK101 were carried out. The blots were probed with a carQRS specific probe. The resulting autoradiograph revealed that the three strains, UWM300-2, each had the correct predicted genotype (Figure 7.5).

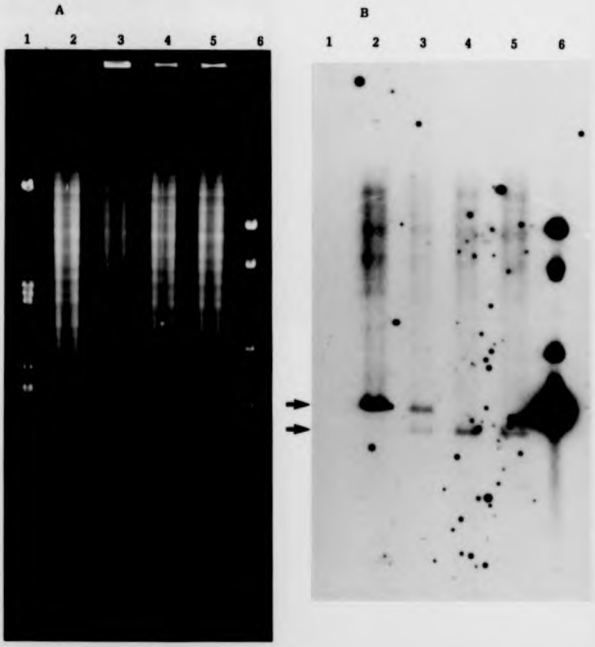
7.3 Activity of car-related genes in a carS$^-$ background:

7.3.1 UWM301::pDAH217:

M. xanthus strain UMW301 was transduced using the bacteriophage, P1, with the plasmid pDAH217. This plasmid encodes a fusion between p^{QRS} and a promoterless *lacZ* gene (Hodgson, in press). Upon integration into the chromosome, levels of β-galactosidase produced by the *lacZ* gene

Figure 7.5 Restriction digest and blot of chromosomal DNA of two carS⁻ strains: Chromosomal DNA was obtained from strains DK101 (lane 2), UWM300 (lane 3), UWM301 (lane 4) and UWM302 (lane 5), and was digested with MluI before being run on an agarose gel (panel A). Lambda DNA digested with PstI (lane 1) and plasmid pDAH191 (Hodgson, in press) digested with MluI (lane 6) were also run on the gel as controls.

A blot of this gel was probed with the 2261bp MluI/MluI fragment of pDAH191. The resulting autoradiograph (panel B) showed that strain DK101 contained a wild-type, 2261bp fragment (arrowed). Strain UWM300 contained two fragments; a 1971bp fragment encoding a deleted version of carS (also arrowed) and the wild-type fragment. Strains UWM301 and UWM302 each contained only the 1971bp fragment encoding a deleted carS gene.


A
1 2 3 4 5 6
B
1 2 3 4 5 6

may be used to assay the activity of P^{QRS}. The resulting strain UWM301:: pDAH217 was grown under both light-inducing and dark conditions and levels of β-galactosidase were assayed (Figure 7.8).

The results indicate that in the carS⁻ strain, UWM301, the promoter is activated in response to light in a wild-type manner. The carS gene product, therefore, is uninvolved in this activation of P^{QRS}.

The results also show that the level of light-induced expression of the promoter is far larger than the normal wild-type response (the results may be compared with Figure 3.8). In addition, expression of the promoter does not decrease after approximately nine hours as would normally be expected. Instead, expression of the promoter may be split into three distinct phases.

Expression increases greatly from two hours after induction to around nine hours, to reach a level of around 1250 units. This is approximately ten times the light-induced level of expression of this promoter assayed in a wild-type background (Figure 3.8). After this time, the level of expression continues to increase at a lower rate until around 30 hours after induction.

We believe that the increased expression of P^{QRS} may be explained solely by the Car⁻ phenotype of this strain. The carotenoids produced in a wild-type strain would normally block the activating singlet oxygen species produced by the action of light upon protoporphyrin IX. In the absence of carotenoids, this negative feedback is no longer operating and, therefore, P^{QRS} will be continuously activated. It is only after 30 hours when large numbers of cells in the culture begin to die, that expression of P^{QRS} appears to decline. A similar pattern of expression was observed by Robson (1982) using the same plasmid but in a Car⁻, carB mutant strain.

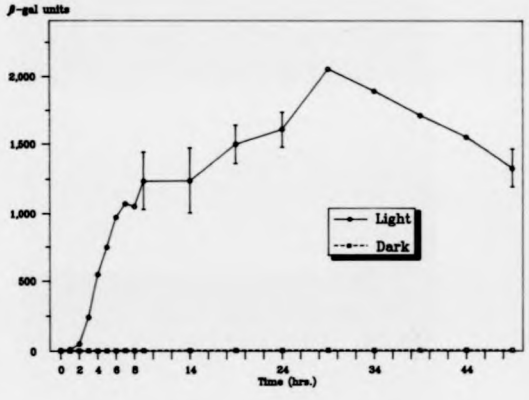


Figure 7.6 β-galactosidase activity of strain UWM301::pDAH217: UWM301, a strain in which the carS gene had been deleted, was transduced with pDAH217. This plasmid encodes P^{QRS} which drives expression of lacZ and integrates into the chromosome by homologous recombination at the carQRS region. The activity of P^{QRS} in this Car^- background may be compared with that in a wild-type background (Figure 3.6).

7.3.2 Activity of *carB* and *carC* in a *carS*$^-$ background:

The above explanation of increased promoter expression assumes that the *carS*$^-$ mutation is responsible for the Car$^-$ phenotype of strain UWM301. It assumes that an activator of carotenogenesis, normally encoded by the *carS* gene, is now absent upon induction of the *carQRS* operon. This assumption was tested by inserting fusions of a promoterless *lacZ* gene with promoters from either *carB* or *carC*, into the chromosome of strain UWM301.

Construction of strain UWM301::pMAR206 was carried out in the same manner as construction of UWM301::pDAH217 - the recipient strain was transduced using P1, with the plasmid pMAR206 encoding a *carC/lacZ* fusion (Fontes M., pers. comm.).

A plasmid in which the *carB* promoter was fused to *lacZ*, however, was unavailable. This fusion was present on the chromosome of *M. xanthus* strain MR401 where the transposon, Tn*5* *lac*, had integrated into the *carB* region (the *carB2* mutation; Balsalobre *et al.*, 1987). A myxophage was used to transduce strain UWM301 with this region of the chromosome. Because strain MR401 is resistant to the myxophage Mx8, a second myxophage, Mx4, was used in this transduction. The resulting strain was named UWM301(ΩMR401::Tn*5* *lac*).

The levels of ß-galactosidase produced by UWM301(ΩMR401::Tn*5* *lac*) and UWM301::pMAR206 were assayed under both light-inducing and dark conditions. The results of these experiments are presented in Figures 7.7 and 7.8 respectively.

7.3.3 UWM301(ΩMR401::Tn*5* *lac*):

Figure 7.7 shows that there is no expression of the *carB* promoter in a *carS*$^-$ background. This is in contrast to expression in a wild-type background, where a steady increase in activity, reaching a maximum after eight hours is observed (MR401; Figure 7.8). This is entirely as predicted and indicates that *carS* encodes an activator of *carB*. In the absence of the CarS protein, the gene(s) at *carB* remain unexpressed and a Car$^-$ phenotype results.

Interestingly, it would seem that the unregulated promoter exhibits a higher level of activity than its normally regulated but uninduced counterpart. The levels of activity measured in both the light and dark for strain UWM301(ΩMR401::Tn*5* *lac*) are both approximately double the level observed for the same promoter in the dark in a wild-type background.

This effect, where the removal of the activator of a particular promoter leads to an increase in the background activity of that promoter, has now been observed twice during this study. P^{QRS} in the absence of a wild-type allele of its own activator, *carQ*, also displays an increase in background activity (Section 6.3.3). There is no obvious explanation for these phenomena.

7.3.4 UWM301::pMAR206:

Figure 7.9 shows that in contrast to *carB*, we do observe expression of the *carC* promoter in response to light in a *carS*$^-$ background. The response of this promoter to light in a wild-type background (DK101::pMAR206; Figure 7.10) occurs in two phases. Firstly, expression

increases steadily to reach a maximum of around 50 units, 10 hours after light-induction. As has been previously reported (Fontes and Murillo, 1990) when the culture enters stationary phase, a second far larger increase in activity is observed. This second increase, raises expression of the promoter approximately 20 fold over its level at 10 hours to around 1000 units after 54 hours.

Expression of the *carC* promoter in a *carS*$^-$ background differs quantitatively from that in a wild-type background, but the two phase pattern of expression remains. Instead of the wild-type *carC* expression of around 50 units after ten hours, we observe a steady increase in expression in a *carS*$^-$ background to over 200 units during the same time period. Again, in strain UWM301, expression of the *carC* promoter is greatly increased during stationary phase, reaching levels significantly above those in the wild-type background.

7.3.5 MR148::pMAR206:

As we have previously demonstrated, P^{QRS} in strain UWM301 also displays an increase in activity above the expression in a wild-type background (Section 7.3.1). We believe that the Car$^-$ phenotype and concomitant absence of negative feedback is responsible for this increase in both cases. It was for this reason that we assayed the level of the *carC* promoter in strain MR148 (Martinez-Laborda *et al.*, 1986; Figure 7.11). This strain has a wild-type *carQRS* operon but has a transposon Tn*5* insertion in the chromosome at the *carB* locus (the *carB1* mutation). This strain is *carB*$^-$ and cannot produce carotenoids in response to light. The Car$^-$ strain, MR148::pMAR206, therefore, represents a better control for the above

experiments than the Car$^+$ strain, DK101::pMAR206.

We can see from comparisons between Figures 7.9 and 7.11 that in a Car$^-$ background, light-induced expression of the carC promoter over the first ten hours is unaffected by either the presence or absence of carS. Activity during stationary phase, however, is quite different. The level of activity does not greatly increase as it does in a wild-type background, but instead rises only slightly, to reach a level only just above that at ten hours.

The conclusion we draw from these experiments, therefore, is that the carS gene product does not appear to be needed to activate expression of carC. Certainly over the first ten hours, expression of carC is clearly carS independent. We believe that a separate effect is being observed when we look at the stationary phase carC activity in this strain. This is considered further in Section 7.4.

7.3.6 Identification of carC activator:

In order to assay the activity of the carC promoter in a carQ$^-$ background, we transduced strain UWM303 (Chapter 6) with plasmid pMAR206. Activity was again assayed in both the dark and the light (Figure 7.12). The results show that in this background, expression of carC in the light is not detected, either in the first ten hours, or later during stationary phase. This result would seem to indicate that expression of carC is activated by the carQ gene product. This result, however, was not unexpected. Since we knew that carC was carQRS responsive, then it was to be expected that there would be no carC promoter activity if an element essential for the activity of the carQRS operon, carQ, was absent.

We tested the hypothesis that it was carQ alone that was responsible for the activation of the carC promoter by carrying out a further experiment. We cloned the carQ gene from pSJM3 (McGowan, 1989; Section 5.2) into pMAR206. This was carried out by digestion of pSJM3 with PvuII and EcoRV, followed by insertion of the 953bp fragment generated into the ScaI site in the ampicillin resistance gene of pMAR206. The resulting plasmid was named pSJM147. This plasmid and the original plasmid, pMAR206, were then used to transduce M. xanthus strain MR135. This strain has undergone a deletion and no longer contains the carQRS operon. We have already shown that in this strain, the carQ gene is constitutively expressed due to the absence of the carR encoded regulator (Section 5.4.1).

Using these two strains, MR135::pMAR206 and MR135::pSJM147, the activity of the carC promoter could be assayed in either the presence or absence of the CarQ protein and free from the influence of light. The results of these experiments are presented in Figures 7.13 and 7.14. As expected, there was no carC expression in either the light or the dark in the absence of any of the carQRS operon. In contrast, a high constitutive level of expression was observed in the presence of carQ alone. This experiment confirms that, like P^{QRS}, the promoter at carC is carQ responsive.

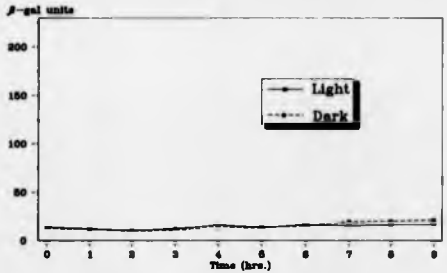


Figure 7.7 β-galactosidase activity of strain UWM301(ΩMR401::Tn5 lac): Strain UWM301, a strain in which the carS gene had been deleted, was transduced with part of the chromosome of strain MR401. In strain MR401, Tn5 lac has integrated into the chromosome within the carB region (the carB2 mutation). The carB promoter, therefore, drives expression of lacZ.

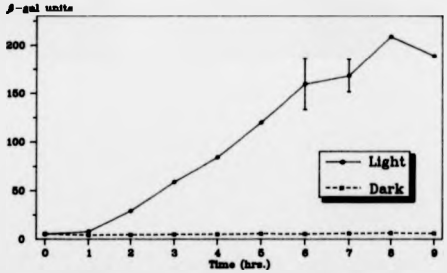


Figure 7.8 β-galactosidase activity of strain MR401: The carB promoter drives expression of the lacZ gene in a wild-type background. These results confirm previously published results involving the same strain (Balsalobre et al., 1987).

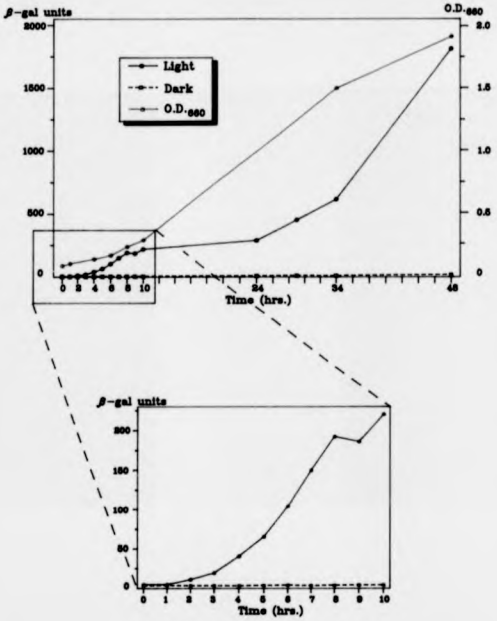


Figure 7.9 β-galactosidase activity of strain UWM301::pMAR206: Strain UWM301, a strain in which the carS gene had been deleted, was transduced with pMAR206 (Fontes M., pers. comm.). This plasmid encodes the carC promoter which drives expression of lacZ.

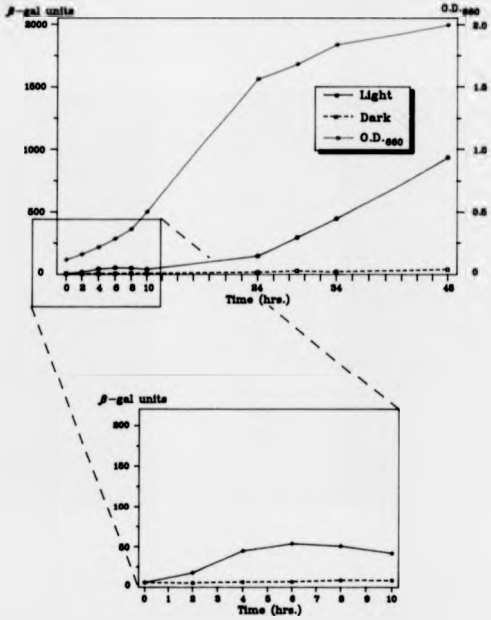


Figure 7.10 β-galactosidase activity of strain DK101::pMAR208: The carC promoter drives expression of lacZ in a wild-type background. The large increase in carC expression as the culture enters stationary phase confirms previously reported results (Fontes and Murillo, 1990).

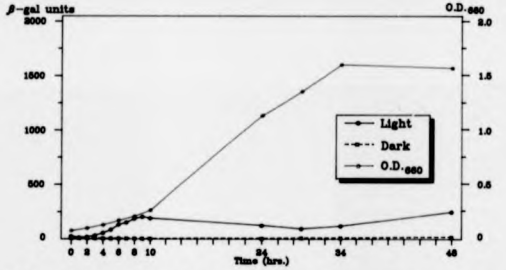


Figure 7.11 β-galactosidase activity of strain MR148::pMAR206: Activity of the carC promoter was assayed in strain MR148. This strain encodes a wild-type carQRS region but is Car⁻ due to a transposon insertion within the carB region (the carB1 mutation).

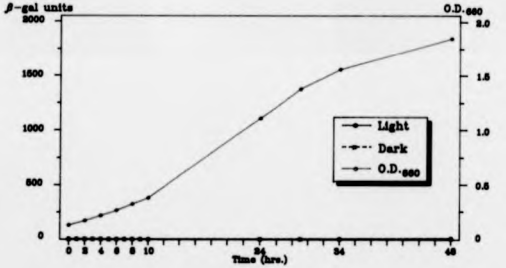


Figure 7.12 β-galactosidase activity of strain UWM303::pMAR206: Activity of the carC promoter was assayed in the carQ⁻ background of strain UWM303 (Chapter 6).

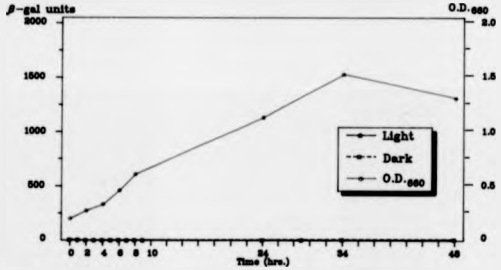


Figure 7.13 β-galactosidase activity of strain MR135::pMAR208: The activity of the carC promoter was assayed in strain MR135, a strain in which the wild-type carQRS region had been deleted.

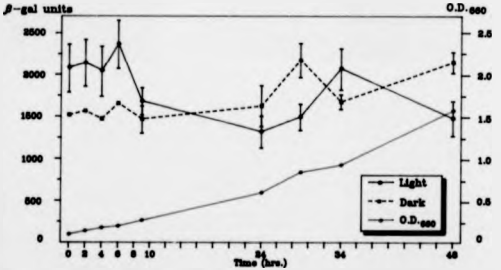


Figure 7.14 β-galactosidase activity of strain MR135::pSJM147: The activity of the carC promoter was assayed using a modified version of plasmid, pMAR208. This plasmid was modified by the insertion of P^{QRS} driving expression of carQ.

7.4 Discussion:

7.4.1 carQ activation:

The experiments presented in this chapter have addressed, not the regulation of expression of the carQRS operon, but the activator known to be encoded by this region, of other car-related genes. We have shown that two activators of these other genes are produced by the carQRS operon. The genes at carB are activated by the carR gene product, whereas those at carC are activated by carQ.

Activation by the carQ gene product has now been demonstrated for two separate promoters - those of the carQRS operon (Chapter 5) and carC. Since we do not know the nature of this activation by the CarQ protein (Section 5.8), clearly an analysis of the DNA sequence of both promoters for similarities could increase our understanding of the process. This analysis was attempted using preliminary carC sequence data (Murillo F. and Fontes M., pers. comm.), however, no homology was observed (data not shown). Further analysis awaits the identification of the start of transcription of carC by the above workers.

7.4.2 Strain MR466:

Combining the results obtained in this chapter and the model developed in Chapter 5, we may now explain other observations about the regulation of the car-regulon. We noted in Section 4.1.1 that strain MR466 displayed constitutive expression of the carB promoter, but that it remained Car^+.

The mutation responsible for this phenotype has been shown to have occurred within the carS gene. It is now apparent that this strain does not display a Car^C phenotype, because although carB is affected by the mutant CarS protein, carC is CarS independent. Constitutive expression of carB will lead to the accumulation of phytoene. Regulation of expression of the carC activator carQ, however, remains wild-type in this strain. Without the carC encoded enzyme phytoene dehydrogenase, therefore, carotenogenesis will not take place in the absence of light-induction.

7.4.3 Transposon insertion ΩDK1911:

Expression of carC was also assayed in a strain in which transcription of the carQRS operon had become constitutive due to the insertion of a transposon (ΩDK1911 in strain DK2727; Martinez-Laborda et al., 1986). This insertion caused constitutive expression of carB but did not affect the photo-inducible expression of carC (Fontes and Murillo, 1986). It was also reported that upon light-induction of this strain, expression of carC was an order of magnitude above wild-type.

Because carS encodes an activator of carB, constitutive expression of carS due to the transposon insertion would lead directly to the observed constitutive expression of the carB promoter. The carC promoter, however, is activated by the product of the carQ gene. Although carQ is also constitutively expressed, the model states that all the CarQ protein will be sequestered to the membrane by the carR gene product. In the dark, therefore, none is available to activate the carC promoter and hence we observe no carC expression.

Upon light-induction, the model states that sequestration by the CarR

protein ceases. This would lead to a large amount of the CarQ protein, immediately becoming available within the cell and hence to a very rapid induction of expression of the carC promoter. Such a rapid induction would give rise to the greatly increased expression above wild-type observed.

7.4.4 Car phenotypes of strains MR466 and DK2727:

A problem arises when we compare the Car phenotypes of the two above described strains. Both have been shown to be phenotypically $carB^{C}/carC^{+}$. Strain MR466, however, is Car^{+}, whereas insertion of the transposon ΩDK1911 leads to an orange, weakly Car^{C} phenotype (Martinez-Laborda et al., 1986). The latter phenotype is similar to that of another strain in which expression of the carQRS operon is driven by different promoter (the promoter of the kanamycin resistance gene of Tn5; Section 1.7.4).

An explanation for this apparent anomaly lies in an analysis of the carotenoid content of each of the two strains. Both strains would accumulate large amounts of the first C_{40} precursor in carotenoid synthesis, phytoene, due to the activity of the expressed carB products. This would not be detected as a colour change however, because phytoene is colourless. Neither strain is able to produce the first red coloured carotenoid, lycopene, because this is produced by the action of phytoene dehydrogenase expressed from the carC gene. In strain MR466, expression in the dark of the carC activator, carQ, is driven only at the weak background level. The carC gene is, therefore, not expressed in this strain and the cells remain yellow in the dark. Similarly, expression of carC in strain DK2727 is prevented by sequestration of the CarQ protein as discussed above.

Analysis of the carotenoid content due to the insertion of the transposon ΩDK1911 reveals it to be similar to that of the weakly Car^C strain MR7 (Murillo F., pers. comm.). This strain contains the carA1 mutation in the carB linked gene, carA. Although a large amount of phytoene is accumulated, small amounts of the various intermediates between this compound and lycopene are also detected (Martinez-Laborda et al., 1990). These intermediates, especially neurosporene, have been implicated in causing the weak orange colour of the Car^C phenotype. Their presence has been attributed to the effects of an unspecific dehydrogenase activity, probably encoded by a gene(s) within the carB region.

One explanation for this apparent anomaly may lie in the nature of the carS encoded activator of the carB region. It is possible for example, that the truncated CarS protein of MR466 is only able to activate those genes responsible for the synthesis of phytoene. Its wild-type counterpart in strain DK2727 may, in addition, be able to activate the gene(s) responsible for this non-specific dehydrogenase activity and, therefore, cause the orange phenotype.

An alternative explanation lies in the level of expression of carB caused by the two carS alleles. The insertion of the transposon ΩDK1911 has been shown to cause the greatest expression of carB of all the mutations linked to carQRS (Balsalobre et al., 1987). In contrast, the carS1 mutation causes expression of carB at levels far below this, albeit constitutively (Murillo F., pers. comm.). It is possible, therefore, that in strain MR466, the carB expression is simply not sufficient to cause the accumulation of significant amounts of phytoene and/or the proposed non-specific dehydrogenase.

7.4.5 Stationary phase expression of *carC*:

The large increase in expression of *carC* during stationary phase is *carS* independent - it occurs in both *carS*$^-$ (UWM301) and *carS*$^+$ (DK101) backgrounds. It would appear that this increase is dependent upon activity of the gene at *carB* into which the Tn*5* transposon in strain MR148 has integrated. In the presence of this *carB1* mutation, the stationary phase increase does not take place. We have already demonstrated using strain MR401, however, that expression at *carB* is activated by the *carS* gene product (Section 7.3.3). How then can the large *carB* activated increase in stationary phase *carC* expression take place, in the *carS*$^-$ strain, UWM301::pMAR208 (Figure 7.8), if the activator of *carB* itself is absent?

A simple explanation would be to assume that there are at least two independent promoters within the *carB* region. One is assayed by the fusion to *lacZ* in MR401, while the second drives expression of a gene that is interrupted by the separate transposon insertion in MR148. This is supported by evidence that several genes are encoded by the *carB* locus (Martinez-Laborda and Murillo, 1989) and that they are driven by at least two light-inducible promoters (Ruiz-Vazquez and Murillo, 1990).

We would suggest that expression of the apparent stationary phase *carC* activator interrupted by the *carB1* mutation in MR148, cannot be *carS* responsive. This would mean that not only are there several promoters within the *carB* region, but that they are separately regulated. Replacement of the inserted Tn*5* in strain MR148 with Tn*5 lac*, has not been reported and expression of this region of the chromosome cannot yet be assayed. Work to confirm this hypothesis, therefore, awaits the construction of such a strain.

Chapter 8.

Further Sequence of the carQRS region.

8.1 Introduction:

When the carQRS region of M. xanthus was sequenced (McGowan, 1989), the open reading frame of another gene, encoded downstream from carS, was also revealed. This gene, named ORFY, was located on the opposite strand from, and transcriptionally convergent with, carS. The product of this gene was thought unlikely to be involved in carotenogenesis. Only part of this gene had been sequenced, however, and we did not know either the position of the start codon of this gene, or the sequence of its promoter. We wished to extend the sequence of the carQRS region, therefore, in order to identify both these elements.

In addition, ORFY is transcribed convergently with respect to carS and the two translational termination codons are separated by only 21bp's. It is possible that the 3' ends of each of the relative mRNA transcripts overlap. This raises the possibility that some form of interference of translation by 'antisense RNA' may occur. Similarly convergent genes in M. xanthus have been previously reported, where the coding regions of csgA and fprA are separated by only 8bp's (Hagen and Shimkets, 1990). Again, knowledge of the promoter of ORFY and the study of its activity, could become factors in understanding aspects of the regulation of carotenogenesis by the carQRS operon.

8.2 Sequencing strategy:

We sequenced the XhoI/SalI fragment adjoining the carQRS region at position 3134. This fragment was sub-cloned from pDAH191 (Hodgson, in press), into M13 mp18 digested with SalI and single stranded DNA from

the resulting phage was sequenced. We also generated a series of fragments with random end-points, by subjecting the same fragment to sonication. These fragments were ligated into M13 mp10 digested with SmaI. Thirteen of these fragments were sequenced in both orientations, to produce a contiguous sequence from the XhoI site to the SalI site. Some of these fragments were resequenced using dITP in place of dGTP in order to overcome problems caused by compressions on the gels.

In addition, using the sequence already generated, we cloned into M13 mp10 digested with SmaI, the NaeI/NaeI fragment of pDAH191 from position 3051 to position 3347. This enabled us to sequence across the junction between the sequence generated and the known sequence of the carQRS region. The resulting sequence confirmed that the two were indeed contiguous.

8.3 Analysis of sequence generated:

The final sequence enabled us to add a further 668bp to the known sequence of the carQRS region (Figure 8.1). The start codon of ORFY was identified. This open reading frame starts at an ATG codon at position 3213 and ends at a TGA codon at position 2705. From its derived amino acid sequence, it is believed to encode a protein of 170 amino acids with a molecular weight of 18054.

In addition, the 3' end of another open reading frame, upstream from and on the same strand as ORFY, was also identified within this region. The start of this open reading frame lies beyond the SalI site at the end of the sequence. The stop codon, TGA, lies 53bp upstream of the start of ORFY at position 3268. From the derived amino acid sequence, 178 amino

acids are encoded by this sequence with a molecular weight of 18713. We propose to name this open reading frame, ORFZ.

The OWL data base, Seqnet, Daresbury Laboratory, was screened for proteins with homology to the amino acid sequences of the two open reading frames, however, no significant similarities were found.

The structure of this region (with the stop codon of the upstream gene, ORFZ, positioned only a small distance from the start of translation of ORFY) strongly indicates the co-transcription of these genes. If this were indeed the case, then we still do not have the promoter sequence of either gene. With this possibility in mind, further work in this particular area was abandoned.

```
      3140        3150        3160        3170        3180        3190        3200
....CCGGGTG ACCCCCCTCC ACCCCCTCCG CGTTCCCCAG CTTCCACTTC CTACCCACCT CCTCCCCCGA
....ArgGlu GlyGlyGlnL euProGlnAl aAsnAlaLeu LysTrpLysA rgArgLeuGl uGlnProSer

      3210        3220        3230        3240        3250        3260        3270
CACGTCCTCA GACATGTCCG CACCCTACCC CTCCCCCCCG CCAGAGTCGG AGCCGACAAC GCCCCTCAGT
ValHisGluS erMet  <-- ORFY                                          HETTy

      3280        3290        3300        3310        3320        3330        3340
AGGACCCCGT GATCCCCCAG TCCAGACTCG AGTACGTCTC CCCCCCCACG CCGCCCTCAT CCCACCCCAG
rSerAlaThr IleGlyTrpH isLeuSerSe rTyrThrGlu AlaAlaValG lyGlyGlnAs pTrpGlyLeu

      3350        3360        3370        3380        3390        3400        3410
CTTCGTCCCG CCCCCCGAGA ACGTGTCCTG GAACCCCGAC ACCCGAAAGC CGACCCAGTA ACCGAACCCC
LysThrGlyA laGlySerPh eThrAspGln PheArgSerL euArgPheAr gValSerTyr GlyPheProG

      3420        3430        3440        3450        3460        3470        3480
CCCCAGATGT CCCCCCCGAC CCCCAGCTCC CCCTCCCAGC CCAACGTCGA CACCGACGTG CCGTAGTCCC
lyTrpIleAs pGlyGlyVal GlyLeuGlnA laGluTrpGl yPheSerSer ValSerThrG lyTyrAspSe

      3490        3500        3510        3520        3530        3540        3550
TGACCTCCCC GTCCAACCCC CCCCTCCCCG CCCCCACCCC CTTCCCCCCC TTCCCCCTCA CCAAGAACTG
rValGlnAla AspLeuAlaA laSerGlyAl aGlyLeuGly LysGlyProL ysProSerLe uPhePheGln

      3560        3570        3580        3590        3600        3610        3620
ACCCCCCCCC TCCAGCCCGA CCCACCCCAC CACCCCCACC GACACCTCCA ACCCCACCCC CCCCAACAAG
GlyAlaGlyG luLeuArgVa lTrpArgVal LeuProValS erValGluLe uGlyValAla ProPheLeuA

      3630        3640        3650        3660        3670        3680        3690
CCCTCCCTCC CCCTCAGCCC GCTGTTCCAG CACCCCTCCA CCTCCACACC CCCCCTCAGA ACCCCCCCCC
rgGlnThrGl yThrLeuPro SerAsnSerS erAlaAspVa lAspPheAla ArgThrLeuL euGlyAlaAr

      3700        3710        3720        3730        3740        3750        3760
CCACCCCCCC ATCCCCCAGC CACCCCTTCC CCTCCCCCAT CCCCAAGAAG TACCCCTACA CCCCCTCCCC
gLeuGlyAla HisGlyLeuS erAlaAsnAl aGluGlyMet GlyPhePheT yrArgTyrLe uAlaGlnAla

      3770        3780        3790        3800
CCTCACCACC GACTCCCTCC CCACCCCCTC CCCCCTCCTC GAC....
ThrLeuVal SerAspThrAl aValAlaGlu ArgThrThrS er....   <-- ORFZ
```

Figure 8.1 The DNA sequence of the XhoI/SalI fragment adjoining the 3' end of the carORS region: The 5' end of this sequence is contiguous with the 3' end of the carORS region sequence (McGowan, 1988). The derived amino acid sequences of ORFY and the partial open reading frame, ORFZ, both encoded by the lower strand (not shown), are also included.

Chapter 9.

Concluding Remarks.

8.1 Concluding remarks and the future:

From an initial description of the carQRS operon, we now have a far better idea of the series of events involved in the regulation of carotenogenesis. Although some of the steps remain to be proven, we may now describe this pathway from the initial reception of a light stimulus, through the initiation of transcription of the carotenoid synthesising genes at carB and carC, and the production of carotenoids:

1) Light with a wavelength of 410nm is absorbed by protoporphyrin IX, causing the latter to be excited to the triplet state ($PPIX^1$).

2) $PPIX^1$, itself toxic, can cause the formation of the long-lived and highly cell damaging singlet oxygen species.

3) Singlet oxygen is believed to inactivate the membrane bound CarR protein, possibly by triggering its proteolytic destruction.

4) In the absence of CarR, the formerly sequestered CarQ protein is free to activate P^{QRS} and cause transcription of the carQRS genes.

5) The gene products of carQ and carS activate transcription of carC and the carB gene cluster respectively. Both the mechanism of this activation, and the mechanism of activation of P^{QRS} by CarQ, remain poorly understood.

6) The carotenoids, formed by the carB gene cluster and carC gene products are present within the membrane. They quench both $PPIX^1$ and singlet oxygen. The pathway is subject, therefore, to negative feedback control. When the cell is returned to the dark, the translational coupling of the genes allows the rapid re-establishment of sequestration of CarQ by CarR.

The carQRS operon remains an extremely interesting area of study and indeed, some of the future experiments suggested within this thesis are the subject of current research projects. Research is underway into the structure of the carQRS light-inducible promoter. This thesis has indicated the minimum region upstream of the carQ transcription start site required for P^{QRS} expression to be 145bp. Work is now underway in an attempt to determine which parts of this region are responsible for this activity.

Firstly, the effect of mutagenesis of this region, by the random insertion of 10bp oligonucleotides, is being determined. This approach, being attempted by A. Berry, should indicate which areas are worthy of further study by site-directed mutagenesis.

Secondly, attempts are underway to determine whether the carQ gene product can bind to the promoter region and, if possible, the site of this binding. Despite the lack of a "classic" DNA binding motif on the CarQ protein, such an event is a distinct possibility given the requirement of this protein for promoter activity. This work is being attempted by D. Miller, using a gel retardation assay and purified CarQ protein resulting from an expression system.

Speculation as to the role of the carD gene product in the inactivation and possible destruction of CarR by light, is also being addressed. If, as has been suggested, the carD gene product acts as a light-activated protease of CarR, then it would be interesting to discover whether CarR is still present in the light in a carD$^-$ background. This experiment is currently underway using antibodies directed to a CarR/ β-galactosidase protein fusion.

Finally, work is also underway in an attempt to determine the cellular location of CarQ. This work, still in its early stages, involves the

modification of the CarQ protein to include a series of amino acids that are recognised by an antibody. The modified protein will then be introduced into the CarQ$^-$ strain, UWM303 (Chapter 8), in order to determine whether this modification upsets the normal function of CarQ. Assuming that it does not, the ability to determine the location of this protein using the antibody can then be used to illustrate the sequestration of CarQ to the membrane in the dark predicted by the model described in Chapter 5.

Appendix 1.

Appendix 1:

This program, used to work out beta-gal units, was written in "Mallard" BASIC for the Amstrad, PCW9512:

```
10 PRINT CHR$(27);"E":PRINT CHR$(27);"H";
20 PRINT " "
30 count = 1
40 PRINT "                                    LacZ ASSAY ANALYSIS PROGRAM."
50 PRINT " "
60 PRINT "                          PLEASE LOAD PAPER (TO FIRST BAR) & USE PITCH 10 DAISY WHEEL."
70 PRINT " "
80 PRINT "                              PRESS 'ALT' & 'RELAY' TO ENABLE THE NUMBER PAD "
90 PRINT " "
100 PRINT "..........!"
110 PRINT " "
120 PRINT " "
130 INPUT "Please give a title to your data. (eg. strain::construct/growth conditions/date)", TITLE$
140 LPRINT " "title$
150 LPRINT ""
160 PRINT ""
170 PRINT ""
180 PRINT "                              Data for the lacZ assay:"
190 PRINT ""
200 INPUT "time (hrs.) of this sampling point:  ", time$
210 PRINT ""
220 INPUT "vol. (ul) used in lacZ assay (NB. the larger of the two):  ", vol1
230 INPUT "first O.D:  ", od1: INPUT "second O.D:  ", od2
240 avod1 = (od1 + od2)/2
250 INPUT "Blank O.D:  ", bod
260 corod1 = avod1 - bod
270 INPUT "and the time (in mins.) for these O.D.'s:  ", time1
280 REM ------------------------------------------------------------------
290 PRINT " "
300 INPUT "second volume (ul) used at this sampling point:  ", vol2
310 INPUT "first O.D:  ", od3: INPUT "second O.D:  ", od4
320 INPUT "and the time (mins.) for these O.D.'s:  ", time2
330 corbod = (vol2/vol1)*bod
340 avod2 = (od3 + od4)/2
350 corod2 = avod2 - corbod
360 REM ------------------------------------------------------------------
370 PRINT " "
380 PRINT "                              And now data for protein assay:"
390 PRINT " "
400 IF count >= 2 THEN GOTO 420
410 INPUT "average volume (ul) cells used during protein assay:  ", provol
420 INPUT "for first volume, amount of protein (ug/ul) from standard curve:  ", pro1
430 INPUT "for second volume, amount of protein (ug/ul) from standard curve:  ", pro2
440 avpro = (pro1 + pro2)/2
450 REM ------------------------------------------------------------------
460 Bgal1U1 = 1000*233.333*provol*corod1/(time1*avpro*vol1)
470 Bgal1U2 = 1000*233.333*provol*corod2/(time2*avpro*vol2)
480 avBgal = (Bgal1U1 + Bgal1U2)/2
490 PRINT " "
500 INPUT "Are these figures correct (Y or N)? ", figs$: figs$=UPPER$(figs$)
510 IF figs$ ="N" THEN GOTO 530
520 IF figs$ ="Y" THEN GOTO 540
530 PRINT CHR$(27);"E": PRINT CHR$(27);"H": GOTO 180
540 count = count + 1: PRINT " "
550 LPRINT "Bgal units at "time$" hrs = "ROUND(avBgal,2)
560 LPRINT ROUND(Bgal1U1,2)"units in"vol1"ul sample, and"ROUND(Bgal1U2,2)"units in"vol2"ul sample.
570 PRINT " would you like to:"
580 PRINT "1) repeat the calculations for a different time point?"
590 PRINT "2) repeat the calculations fora new set of data?"
600 INPUT "(answer 1 or 2 & press RETURN)"; answ$: answ$=UPPER$(answ$)
610 IF answ$ ="1" THEN PRINT CHR$(27);"E":PRINT CHR$(27);"H": GOTO 200
620 IF answ$ ="2" THEN PRINT CHR$(27);"E":PRINT CHR$(27);"H": LPRINT "": LPRINT "": GOTO 130
```

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