A Thesis Submitted for the Degree of PhD at the University of Warwick

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Carotenoid Regulation in *Myxococcus xanthus*

Samantha Jane Bryan Bsc (Hons) Leics

*A thesis submitted for the degree of Doctor of Philosophy in the University of Warwick.*

Department of Biological Sciences

September 2003
Dedicated to:

This thesis is dedicated in loving memory of Brenda Irene Bryan and Barbara Mary Faulkner. Two angels who have returned to heaven, you both suffered so much through your constant battle with cancer, yet never once complained. You both have been a constant source of inspiration to me, never giving up hope that one day you would beat the cancer. You are my heroes and I know that if I progress to being half as brave and selfless as you, I will have achieved something great. May you both rest in peace and this is for you both, thank you for inspiring me to keep going right to the end.
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Declaration

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

..........................................................  
Samantha Jane Bryan

I certify this statement to be correct.

..........................................................
David A. Hodgson
Abstract

The Gram-negative soil dwelling bacterium *Myxococcus xanthus* synthesises carotenoids on exposure to UV illumination. These pigments give the colonies a distinctive orange/red colour, as well as affording them valuable protection from high energy species, generated by chemical reactions fuelled by light. Genetic dissection of the regulation of carotenogenesis has allowed elucidation of transduction of the light signal to the carotenogenic machinery within the cell.

The key element in the carotenogenic regulon is the genetic switch manifested by CarR and CarQ. CarR is an integral membrane protein (anti-sigma factor) which sequesters CarQ, the sigma factor, to the membrane in the dark. When cells are illuminated the photosensitiser protoporphyrin IX becomes excited, being unstable it transfers its excitation energy to molecular oxygen. This generates the high energy species singlet oxygen, which is capable of causing severe cellular damage. Singlet oxygen interacts with CarR possibly through the mediation of CarF, the net result is the destruction of CarR and the release of the sigma factor CarQ. CarQ then mediates transcription from the *carQRS* promoter and the *crtl* promoter. Transcription from the *carQRS* promoter leads to the generation of more CarQ and CarS, CarS causes de-repression of the *crtEBDC* operon. The carotenogenic enzymes encoded by *crtl* and the *crtEBDC* cluster catalyse the production of carotenoids, which quench the initial signalling molecules singlet oxygen and protoporphyrin IX. CarR levels accumulate and CarQ is once again secured in an inactive state. This provides a nice example of negative feedback.

This work investigates the interaction of CarQ with its cognate promoter at *carQRS* through *in vivo* and *in vitro* molecular and genetic techniques. Site directed mutations were assessed *in vivo* through the use of lacZ transcriptional fusions, this allowed the identification of important regions in the *carQRS* promoter. The interaction between the *carQRS* promoter and the divergent *gufA* promoter was also assessed. *In vitro* experiments were used to attempt to further characterise individual mutations.

The negative feedback loop was assessed in a *crtl* mutant to define whether *crtl* was subject to autoregulation. Previously identified genes downstream of *crtl* were mutated to allow phenotypic analysis and identification of putative roles in carotenogenesis.
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$^1\text{O}_2$</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>$^3\text{O}_2$</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>$^3\text{PPIX}$</td>
<td>Triplet protoporphyrin IX</td>
</tr>
<tr>
<td>A-motility</td>
<td>Adventurous motility</td>
</tr>
<tr>
<td>ap</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>aph</td>
<td>Apramycin cassette</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair(s)</td>
</tr>
<tr>
<td>Car$^r$</td>
<td>Unable to synthesize carotenoids</td>
</tr>
<tr>
<td>Car$^c$</td>
<td>Constitutive expression of carotenoids</td>
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<tr>
<td>Car</td>
<td>Carotenoids</td>
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<td>carQRS</td>
<td>Region of DNA encoding carQ, carR and carS</td>
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<td>Dithiothreitol</td>
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<tr>
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<td>Phytene dehydrogenase</td>
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<tr>
<td>PPIX</td>
<td>Protoporphyrin IX</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>Amino acid</td>
<td>Three-letter abbreviation</td>
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<tr>
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<tr>
<td>Alanine</td>
<td>Ala</td>
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<tr>
<td>Arginine</td>
<td>Arg</td>
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<td>Valine</td>
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1. Introduction

1.1. The Myxobacteria

Myxobacteria are Gram-negative unicellular rod shaped bacteria that can occur in virtually any habitat. The order of Myxococcales is divided into the sub-orders of Cystobacterineae and Sorangineae. The genus *Myxococcus* has several species the four main ones being *M. xanthus*, *M. fulvus*, *M. virescens* and *M. stipitatus*. The German botanist H.F.Link identified the first Myxobacterium *Polyangium vitellinum* in 1809. Then in 1857 M.J. Berkley discovered and named two more species *Stigmatella aurantiaca* and *Chondromyces crocatus* which he classified as Hyphomycetes. In 1892 the prominent mycologist Roland Thaxter, identified these species as Myxobacteria and defined their lifestyle while describing them as being a “curious and disagreeable little thing” (Thaxter, 1892).

Generally Myxobacteria can be found on the dung of herbivores, decaying plant matter and the bark of trees (Reichenbach and Dworkin, 1991). They are mesophilic soil micro-organisms with a temperature optimum of 30°C. They are global colonisers surviving in a vast array of environments including the tropical rain forests of Brazil (Ruckert, 1972), central European forests (Dawid, 2001), peat bogs (Dawid, 2001) and hot and cold biotopes such as the deserts in Arizona and the Antarctic (Dawid, 2001). They have even been found in aquatic environments including saline biotopes such as splash water zones (Dawid, 2001). Several
endearing features of the Myxobacteria make them irresistible to researchers. These mainly involve social interactions between individual cells (Dworkin 1996).

Motility in these bacteria is by gliding and comprises two forms, A-motility and S-motility. S-motility is the form associated with social group movement and involves the retraction of type IV pili (McBride, 2001), while A-motility is associated with a more adventurous single celled motility which involves the production and extrusion of slime (Wolgemuth et al., 2002).

Myxobacterial cells can exhibit a swarming motility that allows them to hunt other “prey” bacteria. This has been compared to the pack behaviour seen in wolves (Burnham et al., 1994). Their predatory lifestyle involves the production of lytic exoenzymes, they can then feed on the lysis products of the devoured bacteria which is sufficient to sustain their growth (Dworkin 1962). Myxobacteria are also capable of forming complex three dimensional structures termed fruiting bodies. These morphologically complicated cellular aggregates contain resistant differentiated cells termed myxospores (Dworkin, 1962 and Dworkin, 1996). The production of a population of myxospores within a fruiting body, sometimes within a hardened sporangiole, means that upon relief of starvation a population of germinants is released which are more efficient in thriving in the environment than a single germinating cell.

Fruiting bodies can be generated in all four species of *Myxococcus*, they do however differ in structure and pigmentation. A summary is provided in table 1.1.
<table>
<thead>
<tr>
<th>Species</th>
<th>Fruiting body stalk</th>
<th>Colouration</th>
<th>Other features</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. xanthus</td>
<td>None</td>
<td>Yellow/orange</td>
<td>Bright orange fruits</td>
</tr>
<tr>
<td>M. stipitatus</td>
<td>Stalked</td>
<td>White/fawn</td>
<td>Yellow fluorescence under UV.</td>
</tr>
<tr>
<td>M. fulvus</td>
<td>Constriction at base</td>
<td>White/pink/red</td>
<td></td>
</tr>
<tr>
<td>M. virescens</td>
<td>None</td>
<td>Green/yellow/grey</td>
<td></td>
</tr>
</tbody>
</table>

Table 1-1: Species of the genus Myxococcus and their main similarities/differences.

*M. xanthus* should not be considered a typical member of the myxobacteria, it forms a very primitive fruiting structure compared to other Myxobacteria such as *Stigmatella aurantiaca* (Figure 1.1). It does however possess some unique features of its own. For example it is the only myxobacterium for which illumination inhibits fruiting rather than being a prerequisite for fruiting. It was also one of the first prokaryotes in which, a new class of retroelements were identified, called retrons, which encode reverse transcriptases (Dawid, 2001).
Figure 1-1: The generation of different fruiting bodies among the order Myxobacteria.

A- *M. fulvus*  B- *M. stipitatus*  C- *Stigmatella aurantiaca*  D- *Chondromyces crocatus*

Structures vary among individual species ranging from the very simple *M. fulvus* to the elaborate structures generated by *C. crocatus*. Photographs courtesy of Hans Reichenbach. (Http://www.microbiology.med.umn.edu/myxobacteria/index.html)
1.2. *Myxococcus xanthus*

Most researchers who study Myxobacteria choose *M. xanthus* (Figure 1.2). This is because it is easy to culture and manipulate has several generalised transducing phages and is susceptible to coliphage P1 attachment. This allows intergenic transfer between *E. coli* and *M. xanthus* (O'Connor and Zusman, 1983). *M. xanthus* can also be electroporated and is readily studied using *lacZ* reporter genes. They can also support the introduction of transposons (Gill and Schimketts, 1993).

*M. xanthus* exists in two forms, in the vegetative mode of growth, cells are typically rod-shaped (0.7-1.2 by 3-12μm), with a doubling time of ~5 hours. When conditions become unfavourable due to nutrient limitation, around 100,000 cells aggregate to form a mound of cells termed a fruiting body, within which a subset of cells form resistant myxospores (Dworkin, 1996). During the formation of these resistant structures, some cells are sacrificed so that a minority can become stress-resistant spores that germinate under favourable conditions (Dworkin and Kaiser 1993). Some Myxobacteria though cheat this process. Experimental data have shown that when genotypes deficient for fruiting body development (including several lines that evolved for thousands of generations under asocial conditions), are mixed with their developmentally proficient progenitors, then subsequent clones are over-represented among resulting spores relative to their initial frequency in the mixture (Velicer *et al.*, 2000).
Figure 1-2: Fruiting body formation in *M. xanthus*

Fruiting bodies are produced in response to starvation as cells aggregate together through cellular communication (http://www.webpages-uidaho.edu/~hartzell/).
*M. xanthus* also exhibits several characteristics which have previously only been associated with eukaryotes. The presence of serine/threonine kinases (Munoz-Dorado *et al.*, 1991) and conserved ‘eukaryotic’ protein sub-domains (e.g. HMGI(Y) sub-domains of CarD). The genome size of *M. xanthus* is unusually large at 9,454 Kb nearly double that of *E. coli*. Myxobacteria also generate a wide variety of secondary metabolites. *M. xanthus* produces antibiotics such as TA (Varon *et al.*, 1997) saframycin and myxovirescin (Reichenbach and Hofle, 1993). Several Myxobacterial compounds are chlorinated, some contain rare nitro groups. Tartrolan is one of only four known natural products known to complex boron. Epothilon is a paditaxel mimic which stabilises microtubules destroying spindle formation leading to cell apoptosis. Epothilon is produced by *Sorangium cellulosum* yet genes introduced from this operon into *M. xanthus* resulted in the production of epothilones, which are potential anti-cancer agents (Julien and Shah, 2002).
1.3. Motility

Motility is arguably the most impressive feature of microbial physiology. In general, motile prokaryotic organisms move in aqueous environments through swimming, buoyancy control or along surfaces using distinct modes of translocation. Most research has focused on swimming bacteria utilising flagella, but not all swimming bacteria are flagellated such as the cyanobacteria. Rapid swimming of non-flagellated synechococcus was found to be linked to slime extrusion (Hoiczyk and Baumeister, 1998). Swimming is only useful in aquatic environments and many microbes have to survive in harsh environments with low water content and changing humidity. To overcome these unfavourable conditions many prokaryotes employ one of two modes of active cell translocation on a solid surface, gliding or twitching.

1.3.1. Twitching motility

Twitching involves movement of cells in short intermittent jerks of up to several micrometers. It usually requires a moist surface and occurs in a wide variety of bacteria including Neisseria gonorrhoea, Pseudomonas aeruginosa and Escherichia coli, (Henrichsen 1983). In 1980 Bradley proposed that the driving force behind twitching motility in P. aeruginosa was the retraction of polar pili. These being type IV pili which are about 6nm in diameter and up to 4μm in length and located at both cell poles. Indeed active extension and retraction of type IV pili appears to be involved in a wide variety of processes including cell movement (Merz et al., 2000),
conjugation (Yoshida et al., 1999), activation of host cell responses (Merz et al., 1999) and cytotoxicity (Comolli et al., 1999).

1.3.2. Gliding Motility

Historically gliding was defined as the movement of a non-flagellated cell in the direction of its long axis on a surface (Henrichsen, 1972). Several models for gliding have been proposed for different organisms, to explain the seemingly smooth advancement of cells on solid surfaces. These include operation of contractile elements (Burchard, 1981), directional propagation of waves along the cell surface (Humphrey et al., 1989), directional extrusion of slime (Hoicyk et al., 1998) and rotary motors (Pate et al., 1979). Gliding has been predominantly studied in *M. xanthus*, *flavobacterium* and *cytophaga* strain U67.

1.3.3. Myxobacterial gliding motility

Myxobacteria are arguably the most complex of the prokaryotes. When times are hard and nutrients are scarce, thousand of cells swarm together to form complex and elegant fruiting bodies. Within these structures vegetative cells differentiate to form dormant myxospores which can be reawakened at a time when nutrients are more abundant. Early experiments suggested that *M. xanthus* had two independent systems for movements over surfaces, the “S” system for social gliding and the “A” system for more adventurous gliding (Hodgkin and Kaiser, 1979).
1.3.4. Social gliding requires type IV pili

The *M. xanthus pil* genes have been characterised relatively recently and 14 of the 17 genes in the *pil* cluster exhibit sequence similarities with the *P. aeruginosa* type IV pilus proteins. The remaining three, *pilG*, *pilH* and *pill*, have no homologs in *P. aeruginosa*. Mutations in any of these three genes result in loss of pilus formation and S-motility. In addition to the *pil* genes at least one other gene *tgl* (transient gliding), is required for pilus formation and cell movement. Interestingly motility can be restored to *tgl* mutants by non-motile donor cells, although the exact mechanism remains unclear. Pilus retraction may be the driving force behind S-motility (Sun *et al.*, 2000). When wild-type *M. xanthus* glides on a polystyrene surface they occasionally become tethered to the surface and stand on end. Cells from a *pilA* mutant lack pili and do not become tethered. Tethered cells appear to be pulled toward the surface. A model was proposed to explain S-motility, suggesting that a gliding cell repeatedly extends pili from its leading pole (Kaiser, 2000) as the pili retract the cell moves forward (Figure 1.3). Cells can reverse direction by extending and retracting pili from the opposite pole (Sun *et al.*, 2000).
Figure 1-3: Pili-mediated S-motility in M. xanthus (Adapted from Sun et al 2000).

Pilus fragments bind the pili to a solid surface; force is generated by pilus retraction. Gliding involves extruding the pilus fragments forward allowing attachment to a solid surface, followed by retraction of the pili filaments to move closer to the adherence site. Cellular reversals may involve switching active pili bundles from one cell pole to another.
1.3.5. Adventurous Gliding Motility.

This allows individual cells to move over relatively dry surfaces, the mechanism of A- motility was thought to be associated with the extrusion of slime. In cyanobacteria nozzle-like structures were found recently from which slime emanated at the same rate as the bacteria moved (Hoiczyk and Baumeister, 1998). Similar structures have also been located in *M. xanthus* although the nozzles in *M. xanthus* are slightly smaller in diameter (Wolgemuth *et al.*, 2002). This is somewhat remarkable given that the two bacterial groups are evolutionally unrelated. Nozzles in *M. xanthus* are clustered at the poles in a prime position for propulsion and S-motility mutants can still produce slime and follow trails unlike A- mutants, suggesting the presence of slime is a requirement in A-motility. Velocity studies also confirmed that the force of the slime was sufficient to propel the cell forward (Wolgemuth *et al.*, 2002).

The feature of *M. xanthus* biology which concerns the rest of this work is the production of coloured carotenoids as a response to illumination with blue light.
1.4. Biology and light

Light is of fundamental importance to biological systems, indeed most species on earth would cease to exist without the existence of sunlight. It is an integral requirement for photosynthesis and visual perception. Light is also important in bioluminescence, circadian rhythms in a wide variety of organisms, morphogenesis in fungi (Linden and Macino, 1997) and vitamin D synthesis in animals and humans. Light can also be responsible for triggering reproductive cycles for example the algae *Chlamydomonas reinhardtii* requires blue light to differentiate immature pre-gametes into mature gametes (Pan *et al.*, 1996). Light can also be potentially lethal, for example UV light is a potent carcinogen as a result of various modes of photo-induced DNA damage. These include the formation of pyrimidine dimers and hydroxylation of guanosine residues (Kohen *et al.*, 1995). Absorption of radiation in aerobic conditions is also linked to the production of high-energy oxygen species which damage cellular structures irrevocably. To survive this conundrum, organisms have evolved mechanisms of protection against illumination and oxidative damage. These include photoreactivation by photolyases, repair by direct dealkylation, mismatch repair and the SOS response which involves some 20 SOS response genes including *lexA* and *recA* (Humayun, 1998).
1.4.1. Photochemistry and Photosensitisers.

Molecules which absorb light energy have their own intrinsic energy increased as electrons are raised from the ground state to an excited state. Orbitals are always filled in order of energy with the lowest ones being filled first. Hund’s rule states that two electrons in a degenerate set of P-orbitals must be in the ground state in the same orbitals with anti-parallel spins. The second law of thermodynamics also states that a molecule must return to its ground state for maximum stability. This results in a number of possible scenarios. Acquired energy can be released as light energy giving rise to fluorescence, or the energy could be released as heat due to vibrational relaxation (Figure 1.4). The energy can also be released through chemical reactions. Sensitizers are examples of molecules that release energy through chemical reactions with other molecules leading to extensive cellular damage.

When an electron is promoted to a higher energy level and retains the same direction of spin it possessed in the ground level is said to be in an excited singlet state. Once in this state one of the previously paired electrons may alter its direction of spin so that both electrons have the same parallel direction this is termed the excited triplet state. Porphyrins are components of phytochromes and chlorophyll and are examples of sensitizers; the excited triplet state is relatively long lived and can directly interact with proteins and lipids. They can also react with oxygen in a type II mediated reaction which generates singlet oxygen, a species capable of generating huge cellular damage. Carotenoids can disseminate the damage caused by sensitisers by quenching the excess energy and releasing it harmlessly as heat.
Jablonski diagram for photochemical processes. Small boxed arrows represent the direction of electron spin. Absorbed energy may be released as fluorescence, phosphorescence or as a radiation-less conversion. The heavy horizontal lines the singlet energy levels S1, S2, S3 and the ground state S0, and triplet levels T1 and T2. The lighter bars represent the vibrational excited levels associated with each electronic state. The larger arrows show transitions between electronic levels. (Wolken, 1975).

Figure 1-4: Energy diagram showing photochemical processes
1.4.2. Oxidative damage.

Oxygen although required by most organisms to respire can also be extremely toxic and is most potent as a set of energised oxygen-containing species which includes singlet oxygen and peroxide radicals. The sequence of univalent reductions of oxygen is shown below.

\[
\begin{align*}
O_2 & \rightarrow O_2^{•} & \rightarrow H_2O_2 & \rightarrow HO^{•} & \rightarrow H_2O \\
\text{SUPEROXIDE} & & \text{PEROXIDE} & & \text{HYDROXYL RADICAL}
\end{align*}
\]

The most destructive of these is the radical superoxide ion which can attack the macromolecules within a bacterial cell causing mutation or death. Damage to DNA by oxygen radicals is mediated by metal ions especially iron as in the classic Fenton reaction. The other main source of high energy oxygen species is directly through illumination via photosensitisers. Photosensitisation can be mediated by any molecule with \( \pi \) orbitals, but is primarily caused by molecules that contain delocalised electrons, such as those found in tetrapyrolles, aromatics and polyenes, this is because delocalised electrons require less energy to excite than localised ones. Photosynthetic bacteria generate high energy oxygen species as a result of energy capture from light and its subsequent conversion into a proton motive force. \textit{Rhodobacter} sp. have avoided this problem by only engaging in photosynthesis under anaerobic conditions.
1.4.3. Prevention of oxidative damage

The responses to oxidative and photooxidative damage are many and varied. The production of primary antioxidants removes high energy oxygen species. These include chelators, oxygen radical scavengers, superoxide dismutase, catalase and peroxidase. Antioxidant defences are co-ordinately expressed in regulons which typically respond to a particular antioxidant species. The SoxR protein is a regulator of gene expression in response to superoxide radicals (Hidalgo et al., 1998). In *E. coli* the presence of superoxide causes SoxR to activate expression from *soxS* which in turn activates nine other *sox* genes, including MnSOD (manganese-containing superoxide dismutase) and endonuclease IV (Nunoshiba et al., 1992).

The energy generated by visible light (300-600nm) can also be used by photolyases to break cyclobutyl pyrimidine dimers and thymidine dimers restoring the bases to their monomeric form. Binding of the enzyme to its substrate results in a complex, which upon the absorption of light, catalyses the cleavage of the thymidine dimer (Schieferstein and Thoma, 1998). Dealkylation results in the direct reversal of damage by the transfer of a methyl group from the pre-carcinogenic O\(^6\)-methylguanine to a cysteine residue on O\(^6\)-methylguanine–DNA methyltransferase (Kornberg and Baker 1992). Repair of a great variety of damaged and modified bases is achieved by excisions. These can be base excisions, excision of an AP site (abasic site), oligonucleotide excision or excision of an interstrand cross-link (Kornberg and Baker 1992). The mismatch repair system monitors recombination intermediates and depends on seven proteins MutS, MutL, MutH, MutU, exonuclease I, SSB and the PolIII holoenzyme (Fowler et al., 2003). Remarkably MutS can recognise the slight
helical distortion of an incorrect base pair and the DNA can be tracked for great
distances to identify which of the strands is newly synthesised in order to remove and
replace the entire section with high efficiency (Yang, 2000).

The SOS response is induced by DNA damage that blocks replication i.e. damage
form UV, mitomycin or nalidixate (Weigle 1953). In the cell, LexA represses
expression of RecA and the SOS regulon. It is thought that replication cannot
proceed past regions of UV induced DNA damage and that the signal for the SOS
response is ssDNA at the replication fork stalled by UV damage. Both the ssDNA
and the UV irradiated dsDNA are bound by the RecA protein. The activated RecA
complex in turn binds the LexA protein inducing a conformational change which
promotes LexA cleavage and subsequent loss of its repressive activity. The cleavage
of RecA also leads to the self cleavage of the λ-repressor and the UmuD protein
(Bjedov et al., 2003). A complex of the activated UmuD protein with UmuC, RecA
and DNA polymerase III generates a “mutosome” localised at the lesion which
allows the polymerase to replicate past it (Pages et al., 2003). Part of the SOS
response seems to cause modification of DNA polymerases so that they are capable
of replicating past damaged regions of DNA although subsequently introducing
errors at a higher frequency (Humayun, 1998).
1.4.4. Role of carotenoids as photoprotectors

The production of carotenoids is a major subset of responses to singlet oxygen-mediated photo-induced oxidative damage. Carotenoids are secondary metabolites produced mainly in conditions of arrested growth or limited food. They are a large family of yellow, red and orange pigments, which are composed of at least 600 structurally distinct compounds. These consist of a C$_{40}$ backbone which is composed of eight, five carbon isoprenoid subunits. Carotenoids can be mono or bi-cyclic and carry various substitutions. They fall into two chemical classes the carotenes and the xanthophylls, which are oxygen containing carotenes. Carotenoids protect against photosensitisation damage since they are capable of quenching excited photosensitisers, singlet oxygen and are also able to directly absorb incident light. These photoprotective properties are due to an extensive conjugated double bond system, which allows energy quenched from excited species to be dissipate harmlessly as heat. This requires a tract of at least seven conjugated double-bonds (Fiel et al., 1981). The occurrence of carotenoids in the hydrophobic membrane means that they are ideally situated for photoprotection, as they reside in the same environment as the triplet photosensitisers such as PPIX and singlet oxygen which they quench.

In the plant and bacterial photosynthetic reaction centre, carotenoids are positioned to protect the integral chlorophyll molecules from oxidation by singlet oxygen, protect the cell against photosensitisation and to act as an accessory light collecting pigment.
Carotenoids are also produced as a protective measure against illumination with blue light among non-photosynthetic bacteria. In some cases this is independent of light e.g. *Erwinia* (Armstrong *et al*., 1990 and To *et al*., 1994). Light still up-regulates carotenoid production, whereas in others illumination is absolutely required e.g. *M. xanthus*. In some cases carotenoids production can be cryptic, which is the case for *Streptomyces griseus* (Schumann *et al*., 1996).

1.5. Carotenoids

1.5.1. Carotenoids of *M. xanthus*.

*M. xanthus* can synthesise between 50-60 carotenoids but most of these are produced at relatively low amounts. They exhibit extensive unsaturation and may be covalently modified by cyclisation, hydroxylation, ketonation or esterification via sugar residues to fatty acids. The majority of carotenoids found in *M. fulvus* are xanthophylls in the primary form of myxobactin and myxobacton esters and 4-keto-torulene glucoside (Reichenbach and Kleinig, 1984; Hodgson and Murillo, 1993). Carotenoids cause orange/red pigmentation and significant levels are only synthesised upon illumination when levels of protoporphyrin IX increase sixteen-fold (Burchard and Dworkin, 1966; Fontes *et al*., 1993).
1.5.2. Carotenoid synthesis

The pathway of carotenoid production shares a common pathway with steroid and terpenoid production, with condensation of isoprenoid subunits leading to the generation of \( \text{C}_{20} \text{geranylgeranyl-diphosphate (GGPP)} \) (Figure 1.5). Two of these molecules then join to form phytoene, Porter and Lincoln proposed that phytoene was converted to lycopene by a series of four desaturation reactions involving the removal of two hydrogen atoms. This sequentially generates phytofluene, \( \zeta \)-carotene, neurosporene and lycopene and this is certainly true in many fungi and plants. In some bacteria though it is neurosporene and not lycopene that is subjected to hydroxylation cyclisation and other modifications i.e. \textit{Rhodobacter} and \textit{Myxococcus}. The last desaturation step is carried out by a separate dehydrogenase enzyme to phytoene dehydrogenase. This takes place on a previously modified neurosporene molecule (Armstrong \textit{et al.}, 1990).

In \textit{M. fulvus} there is a split in the carotenogenic pathway after the third desaturation of phytoene with neurosporene being dehydrogenated to form lycopene or undergoing hydroxylation to generate hydroxyneurosporene (Figure 1.6). This is converted to a 3,4 dehydro-rhodopin glucoside ester by two dehydrogenations and an esterification. Further modification by cyclisation and oxidation leads to the formation of myxobacton. Lycopene can also be converted to 4 keto-torulene via \( \gamma \)-carotene and 4-keto \( \gamma \) carotene (Kleinig \textit{et al.}, 1975). One of the most abundant monocyclic carotenoids is 4-keto-torulene, the others are made up of the fatty acid esters of 2 glycosylated monocyclic carotenoids myxobactin and myxobacton. These
are also major carotenoids in *Stigmatella aurantiaca* (Kleinig *et al.*, 1970). Myxobacton esters make up 70% of the final carotenoid content. In *M. xanthus* the carotenogenic switch is light, and it is assumed that the pathway is the same as *M. fulvus* yet this has never been proved indeed the presence of 4-keto-torulene has never been detected in wild-type *M. xanthus* strains even in the presence of light (Ruiz-Vazquez *et al.*, 1993).
Figure 1-5: General carotenoid pathway

This depicts the generation of lycopene from geranylgeranyl diphosphate following a series of reactions involving dehydrogenation and cyclization. This pathway varies between individual bacteria and between bacteria and plants (www.genox.com/isa/isa-carotenoids.htm).
Figure 1-6: Pathway of carotenoid production in M. fulvus

Adapted from Hodgson and Murillo, 1993. Enzymatic steps are represented by circles around the functional groups they introduce.
1.5.3. Carotenogenesis in other bacteria.

Carotenoids are ubiquitous and essential components of photosynthetic tissues in plants, algae and cyanobacteria, where they have two major functions. They act as light harvesters and photoprotectors preventing photooxidative damage of the photosynthetic apparatus (Frank and Cogdell, 1996). In Chlamydomonas reinhardtii biosynthesis starts with the condensation of two GGPP molecules to form phytoene; this is catalysed by phytoene synthase. Phytoene is then converted to the coloured ζ-carotene by a two step desaturation reaction catalysed by phytoene desaturase. Further desaturation and cyclisation leads to the synthesis of α and β carotenoids which are then converted to xanthophyll. In Sinapis alba light induction of a phytoene synthase gene was reported, yet transcript levels from other carotenoid genes remained unaltered (Steinbrenner and Linden, 2000). In Haematococcus pluvialis elevated levels of expression in carotenoid biosynthetic genes played an important role in the accumulation of carotenoids. In C reinhardtii both phytoene synthase and phytoene desaturase (which is the rate limiting step in Cyanobacteria), showed increased up-regulation in response to light. Carotenoid accumulation in C reinhardtii seems to be dependent not only on the expression of the corresponding genes but also on the biosynthesis of chlorophyll, the development of the photosynthetic apparatus and the destruction of pigments by photooxidation (Bohne and Linden, 2002).
In *Erwinia uredevora* six carotenoid genes have been found on two operons, *crtEXYIB* and the *crtZ* (Misawa et al., 1990). Genes *crtY, Z* and *X* are responsible for the cyclisation of lycopene, hydroxylation of beta-carotene and esterification to sugars (Figure 1.7). Expression of *Erwinia* GGPP synthase in *E. coli* with *crtM* from *Staphylococcus aureus* generated novel compounds with a C35 backbone. Both carotene cyclases from C40 or C30 pathways accepted and converted the C35 substrate demonstrating the plastic and expansible nature of carotenoid pathways (Umeno and Arnold, 2003). In *Mycobacterium marinum* carotenogenesis is light induced while in *Mycobacterium vaccae* the expression of the carotenogenic genes is repressed in the dark with light relieving this repression (Houssaini-Iraqui et al., 1992).
Figure 1-7: Carotenogenic pathway of Erwinia uredovora.

Adapted from Misawa et al., 1990. Enzymatic steps are indicated and functional groups introduced as a result are circled.
1.5.4. Carotenoid production in plants.

Photosynthesis is purely based on the accumulation of solar energy and its efficient conversion into chemical energy, but this would not be possible without a safety valve, something akin to a lightening rod that dissipates excitation energy in a harmless way. In extreme conditions like scorching dry summers some evergreens while maintaining their light absorbing chlorophyll suspend growth and photosynthesis and dissipate nearly all the light they absorb to prevent further stress (Adams et al., 2001). In plants, carotenoids are primarily in an oxygenated form, xanthophylls. These are synthesised from β-carotene via hydroxylation. While lutein is synthesised from α-carotene, both plants and algae possess an additional set of reactions that rapidly optimise the concentration of carotenoids. Xanthophylls accept excess excitation energy from chlorophyll and readily dissipate the energy as heat (Josue and Frank, 2002). This facilitates the de-excitation of singlet excited chlorophyll, preventing transfer of the energy to oxygen, which could generate singlet oxygen. Carotenoids can also facilitate several backup defence processes including the scavenging of singlet oxygen and triplet state chlorophyll (Adams, 2002). Carotenoids also act as light scavengers at lower frequencies. Indeed when light needs to be used efficiently for photosynthesis the dissipater zeoxanthin is converted to a non dissipating pigment violaxanthin. This is reversed under excessive light (Adams, 1996). Finally carotenoids also act as attractants to birds and insects required by the plant for pollination, they therefore have a minor role in reproduction in plants.
1.5.5. Carotenoids and Humans.

Humans unlike many bacteria and plants are incapable of synthesising their own carotenoids instead they are ingested through the diet. Tomatoes for example, have high levels of lycopenes which have been shown to possess anti-cancer properties (Ras et al., 2000). Oxidative stress and disturbances in cellular redox balances have been identified as key elements underlying a plethora of human diseases including cancer and ageing. Carotenoids can have profound effects on their environment engaging in structural interactions with lipid membranes and proteins (Krinsky et al., 2002). In the retina they bind to the protein tubulin, enhancing visual activity (Bernstein et al., 1997). Zeaxanthin is also preferentially accumulated and incorporated into parts of the mammalian retina. This helps to prevent damage caused by exposure to high irradiation (Landrum et al., 2001). Arrest of the xanthophyll cycle in plants results in an accumulation of zeaxanthin. This is currently being investigated as a possible solution to increase zeaxanthin levels in leafy crops. Beta carotene has also proved useful in HIV patients as it boosts the concentration of CD4 T-cell population. These cells are the primary targets for the virus and numbers are heavily ablated as a result (Coodley, 1993). Research is still ongoing into what role carotenoids play in this replenishing effect. Carotenoids are also used to relieve oxidative stress in Helicoverpa zea. The larvae have a unique yellow colouration in their mandibular glands due to the accumulation of carotenoids. The plants they feed on are rich in carotenoids particularly xanthophyll and lutein. Lutein was detected in the testes, midgut epithelium, body fat and the integument (Eichenseer et al., 2002).
1.5.6. Carotenoid biosynthetic genes in *Myxococcus xanthus*.

In *M. xanthus* the genes involved in carotenoid production are dispersed over three genetically unlinked loci. These are the *carQRS* operon the *crtEBDC* operon and the *crtI* gene. The nomenclature for the Car genes had been updated to these current versions (Botella *et al.*, 1995). The structural genes for carotenogenic enzymes were given the *crt* designation based on their homologies to other carotenogenic enzymes from other organisms. The regulatory genes though have retained their *car* designation with *car* being an acronym for *crt* gene activity regulator genes (Hodgson and Berry, 1998). The table below shows the old and revised nomenclature for the genes involved in carotenogenesis in *M. xanthus* (Botella *et al.*, 1995).

<table>
<thead>
<tr>
<th>Functionality</th>
<th>Old name</th>
<th>New name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene dehydrogenase</td>
<td><em>carC</em></td>
<td><em>crtl</em></td>
</tr>
<tr>
<td>Biosynthetic cluster (orf’s 1-6)</td>
<td><em>carB</em></td>
<td><em>crtEDBC</em></td>
</tr>
<tr>
<td>Geranylgeranyl diphosphate synthase</td>
<td><em>orf1</em></td>
<td><em>crtE</em></td>
</tr>
<tr>
<td>Carotene desaturase</td>
<td><em>orf2</em></td>
<td>-</td>
</tr>
<tr>
<td>Phytolene synthase</td>
<td><em>orf3</em></td>
<td><em>crtB</em></td>
</tr>
<tr>
<td>Hydroxyneurosporene desaturase</td>
<td><em>orf4</em></td>
<td><em>crtD</em></td>
</tr>
<tr>
<td>Neurosporene hydratase</td>
<td><em>orf6</em></td>
<td><em>crtC</em></td>
</tr>
<tr>
<td>Carotene cyclisation</td>
<td><em>orf7</em></td>
<td>-</td>
</tr>
</tbody>
</table>

*Table 1-2: Current and previous designations of carotenogenesis genes in *M. xanthus*. *
The majority of carotenogenic genes are located within the *crtEBDC* operon. This cluster contains eleven open reading frames with potential translational coupling between *orf2* and *orf3*, *orf4* and *orf5*, *orf5* and *orf6*, *orf6* and *orf7*, *orf7* and *orf8*, *orf8* and *orf9* and *orf10*. All the open reading frames except *orf1* appear to be preceded by ribosome binding sites, both *orf3* and *orf9* start with a GTG start codon and not the usual ATG initiation codon. Functions were assigned based on sequence similarities, for example *orf1* was named *crtE* due to its similarity with geranylgeranyl diphosphate synthase (Botello *et al*., 1995). Evidence has suggested that *orf2* encodes an enzyme capable of converting phytoene into phytofluene with *crtI* thought to catalyse the subsequent desaturations to form neurosporene and lycopene. Further evidence supporting this scenario came from assessing the production of intermediates of the carotenogenic pathway, as only phytofluene is generated in a *crtI* mutant (Martinez-Laborda *et al*., 1990). Two distinct regions of *orf9* show homology to ferrochetalase this introduces ferrous iron into PPIX to generate haem. Genes *orf10* and *orf11* are 35% identical and contain putative HTH motifs at their N-terminal regions, with greatest homology to MerR of *Tn501* (Parkhill *et al*., 1998). The other biosynthetic gene *crtI* is unlinked to the *crtEBDC* operon and codes for phytoene dehydrogenase.
1.5.7. Regulation of carotenogenesis in *M. xanthus*

The regulatory genes are located primarily at the *carQRS* locus, except for the *carA* gene which resides next to the *crtEBDC* operon. Another two loci have been found to contain genes required for carotenoid production although these genes have pleiotropic effects and are therefore not strictly carotenogenesis genes. The first of these contains the *carD* gene which has motifs resembling those found in the HMGY(I) proteins in eukaryotes. The second locus (*carE*) contains one of the genes for integration host factor (*ihf*) of *M. xanthus*. Over the last decade research on the carotenogenic loci has elucidated the molecular basis of regulation of the system by utilising a combination of biochemical and genetic means.
1.6. The history of research on carotenogenesis in *M. xanthus*

1.6.1. Identification of the *carQRS*, *crtEBDC*, *carA* and *carC/crtI* loci.

Initial Car⁰ carotenogenic mutants were isolated in laboratories working with *M. xanthus* due to the obvious manifestation of the mutation, which causes bright orange/red colouration due to the constant production of carotenoids regardless of illumination. Conversely very few Car⁻ mutants were isolated since these require cultures to be illuminated to prove that no carotenoids can be made. The carotenogenic loci were originally mapped by the creation of Tn5 insertions which co-transduced to known Car⁰ mutations leading to constitutive production of carotenoids. Five were identified linked to *carR* and one linked to *carA* (Martinez-Laborda *et al.*, 1986).

A further transposon mutant prevented accumulation of carotenoids in both light and dark conditions (Balsalobre *et al.*, 1987). The mutation *carAI* was linked to the *crtEBDC* locus. Tn5 *lac* carries a promoterless *lacZ* gene within Tn5. When this is integrated into the chromosome it provides *in situ* information on the transcriptional activity of the region around the transposon. Tn5 *lac* insertions at the *crtEBDC* operon showed that the locus is only transcribed upon illumination in the wild-type. However in constitutive *carA* and *carR* mutants the locus is transcribed in both the light and the dark. This suggested that expression from the *crtEDBC* locus was orchestrated by a light inducible promoter regulated by the products of both *carA* and *carR*. Tn5 insertions later identified the *carC/crtI* gene as being absolutely necessary for carotenogenesis. The absolute requirement for both the *crtI* gene and the
crtEBDC operon suggested that they must be genes encoding structural enzymes required for the generation of carotenoids or positive regulators.

1.6.2. Genetic dissection of the carR region.

A transposon insertion was linked to the carR region and gave a dark yellow phenotype. This gave rise to the theory that a light activated promoter existed upstream of an activator of carotenogenesis. Screening carR mutants for spontaneous mutants possessing a Car-phenotype generated four mutants that were epistatic over a carR lesion (Martinez-Laborda and Murillo, 1989). One lesion mapped to the crtEBDC operon whereas the others were all linked to carR and these three all abolished expression of Tn5 lac inserted at crtEBDC. Thus linked to carR exists a region which is epistatic over constitutive mutations at carR for the generation of carotenoids. A likely explanation would be that an element at carR has a positive role in expression of the car regulon, but that its activity is repressed or inhibited by carR in the dark.

Sequencing of the entire carR region identified three translationally coupled genes carQ, carR and carS (McGowan et al., 1993). Sequence similarity searches denoted carQ as a predicted sigma factor, carR as an integral transmembrane protein. The carS gene though proved to have no homolog. The regulation of the carQRS locus is strictly dependent on the stoichiometry of CarR to CarQ (Gorham et al., 1996). If for example CarQ is present in a greater number than CarR, expression becomes constitutive. If CarR is more predominant than CarQ, carotenogenesis remains light induced. It was also observed that carR:β-galactosidase fusions expressed in M.
xanthus disappear in the light and that a CarR-protein fusion in *E. coli* was located in the membrane (Gorham *et al.*, 1996). The conclusion drawn from these results was that CarR acts as an anti-sigma factor sequestering CarQ the sigma factor to the membrane in the dark but upon illumination CarQ is released due to an active loss of CarR. Deletion mutants also showed that CarQ is a positive activator of both the *carQRS* and *crtI* promoters and CarS is responsible for activation of the *crtEBDC* locus (McGowan *et al.*, 1996).

### 1.6.3. Localisation of the *Car* structural genes.

Further work analysed the nature of carotenoid production in strains carrying different *car* mutations (Martinez-Laborda and Murillo, 1989). The *carR* strains produced the same carotenoids in both the light and the dark as the wild-type, but the *carA* mutant accumulated phytoene in the dark and wild-type carotenoids in the light. Since *crtEBDC* mutants are unable to produce phytoene and strains carrying a *crtI* lesion accumulate only phytoene, it was postulated that the enzymes required for phytoene production reside at the *crtEBDC* operon and that the gene encoding phytoene dehydrogenase is *crtI*. This was confirmed by subsequent cloning and sequencing (Fontes *et al.*, 1993). Expression studies utilising a *crtI::lacZ* transcriptional fusion proved that *crtI* expression was light induced and that induction was increased when cells were in the stationary phase of growth (Fontes *et al.*, 1993). In the strain carrying the *crtI* lesion more phytoene was produced than the total amount of carotenoids produced in the wild-type strain in the light. This was thought to be due to feedback inhibition by end products in the pathway (Martinez-Laborda and Murillo, 1989).
Further experiments introduced transposons into the \textit{crtEBDC} operon along with transcriptional fusions to a promoterless \textit{lacZ} gene along various sections of the cluster. These data suggested the presence of multiple structural enzymes, which are co-transcribed from a single promoter, which is CarQ-dependent and light inducible (Ruiz-Vazquez \textit{et al.}, 1993). Sequencing of the \textit{crtEBDC} operon unearthed several interesting features. The cluster has eleven open reading frames. The \textit{crtEBDC} promoter showed no homology to the \textit{carQRS} promoter suggesting that CarQ is not the sigma factor governing transcription. It has been suggested that there are two promoters at this operon. The initial promoter being upstream of the first six open reading frames designated \textit{crtEBDC} and is probably transcribed by a vegetative promoter. The last five open reading frames of the cluster form a second operon the (\textit{carA} operon) this is transcribed by a Sig54 dependent promoter (Cervantes and Murillo, 1998).

\section*{1.6.4. Current understanding of the regulon.}

The generation of carotenoids involves the products of three separate loci, which are co-ordinately expressed as a regulon, the \textit{carQRS} operon being the central regulatory locus. Further genes have been implicated in this process. The transcription binding factor CarD, \textit{carE} which encodes integration host factor factor (IHF) and the newly discovered \textit{carF} which is thought to play a role in the destruction of CarR.
1.6.5. The \textit{carQRS} operon

Central to the induction of carotenogenesis is the activation of the \textit{carQRS} promoter and expression of the \textit{carQRS} locus. In the dark there is little expression from the \textit{carQRS} promoter however when cells are exposed to the light, expression increases up to 80 fold (Hodgson, 1993). Sequencing studies have shown that the first open reading frame (ORF) in the \textit{carQRS} locus codes for CarQ, which is a positive regulator of the \textit{carQRS} promoter. Sequence analysis has shown that CarQ belongs to the extracytoplasmic function (ECF) sigma factor subfamily (Lonetto \textit{et al.}, 1994). Thus it is proposed that CarQ directs RNA polymerase to the \textit{carQRS} promoter therefore initiating transcription of \textit{carQRS} (Gorham \textit{et al.}, 1996). Both the CarD protein and IHFA (\textit{carE}) play a role in transcription form the \textit{carQRS} promoter suggesting a complex promoter architecture exists. The second ORF encodes the CarR protein which has a repressive effect on transcription in the dark. It was suggested that CarR acts as an anti-sigma factor, by binding CarQ and preventing its association with core polymerase (McGowan \textit{et al.}, 1993; Gorham \textit{et al.}, 1996). Hydrophilicity plots have suggested that CarR is an integral membrane protein. A CarR-β-galactosidase fusion was found to be unstable in illuminated cells suggesting the light mediated degradation of CarR (Gorham \textit{et al.}, 1996). The final ORF in \textit{carQRS} encodes CarS, a small peptide of 12kDa, which is responsible for inducing carotenoid expression at the \textit{crtEBDC/carA} locus (McGowan \textit{et al.}, 1993; Gorham \textit{et al.}, 1996). When cells are in the dark the \textit{crtEBDC/carA} locus is repressed by the CarA protein (Whitworth and Hodgson, 2001; Cervantes and Murillo, 2002).
Upon illumination and subsequent expression from the carQRS promoter, production of CarS relieves the repression by CarA, by negating its DNA-binding activity and consequently relieving inhibition of crtEBDC/carA (Whitworth and Hodgson, 2001; Lopez-Rubio et al., 2002). In vitro transcription run off assays have demonstrated that CarQ is indeed an RNA polymerase sigma factor that is responsible for the expression of the carQRS locus. Yeast two hybrid experiments have confirmed that an interaction takes place between CarQ and CarR (Browning et al., 2003).

### 1.6.6. The crtEBDC locus

The crtEBDC cluster consists of eleven ORF's, these are arranged sequentially over 12kb. The crtEBDC promoter is regulated by the CarS protein and is therefore light induced (Martinez-Laborde and Murillo, 1989). The first six ORF's are structural genes involved in the early and late stages of carotenoid biosynthesis (Botello et al., 1995). The crtB gene for example encodes a phytoene synthase enzyme. The remaining five ORF's have no known function. Both Orf10 and Orf11 contain putative helix-turn-helix motifs. The CarA protein is encoded by orf10 and binds to a palindrome sequence of AAGGTTnnnnnnAACCTT in the crtEBDC promoter, a GST-CarA fusion was prevented form binding at this site by addition of CarS (Whitworth and Hodgson, 2001). The CarA protein represses the crtEBDC operon in the dark, carA mutants are therefore constitutive and accumulate large amounts of colourless phytoene in the dark. Orf11 was predicted to be a protein similar to CarA suggesting the two may have been duplicated from a common ancestral gene. Deleting orf11 had no effect on the generation of carotenoids or regulation of the crtEBDC promoter. Its function remains elusive (Cervantes and Murillo, 2002).
CarA protein has also been predicted to contain a cobalamin binding domain. Cobalamin is a complex prosthetic group and has been shown to be absolutely required for light activation of the \textit{crtEBDC} promoter regulated by CarA. Data suggest that cobalamin acts as a prosthetic group of CarA that mediates a light activated on-off shift of this protein as a repressor of the \(p^{\text{crtEBDC}}\) promoter (Cervantes and Murillo, 2002).

1.6.7. The \textit{crtI} gene

Sequence homology to other phytoene dehydrogenases suggested that the \textit{crtI} gene encoded phytoene dehydrogenase (Balsalobre \textit{et al.}, 1987) although overall homology was moderate (30-40\%). There was strong sequence homology at the C-terminal and N-terminal domains. Phytoene dehydrogenase catalyses the conversion of colourless phytoene into coloured neurosporene and lycopene strains that are defective in a wild-type copy of the \textit{crtI} gene are \textit{car}- and demonstrate an accumulation of carotenoids in the light. Induction of the \textit{crtI} promoter is dependent on CarQ and light induced expression also seems to be additionally activated by carbon limitation (Fontes \textit{et al.}, 1993). This could be due to the additional requirement for CarD. There is no requirement for \textit{ihfA} at the \textit{crtI} promoter. Sequence analysis also identified similarities between the \textit{crtI} promoter and other Gram negative bacterial promoters. Homology at the -35 region is generally stronger than that at the -10 region. Promoters sharing sequence homology at the -10 region include the \textit{vegA} promoter and the \textit{tps} promoter which are involved in multicellular development in \textit{M. xanthus}. 

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1.6.8. The carD gene.

The protein CarD is involved in regulating at least two distinct processes in *M. xanthus* (Nicolas *et al.*, 1994). It is required for the expression of two different sets of genes that form part of a complex network regulating light induced carotenogenesis. In addition CarD also participates in the starvation induced formation of fruiting bodies. Mutations in *carD* have blocked the progression to mature fruiting bodies as well as the activation of several developmental genes (Nicolas *et al.*, 1994). The CarD protein has homology to the mammalian high mobility group (HMGI (Y)) proteins. A CarD-GST fusion bound to the *carQRS* promoter at a very short DNA sequence that resembled the binding site of HMGI(Y). The HMGI(Y)/HMGA proteins are characterised by an AT-hook motif embedded in a less conserved cluster of basic and proline residues (Aravind and Landsman, 1998). The CarD protein is possibly the only prokaryotic protein with multiple AT hooks (Padamnabhan *et al.*, 2001). HMGI is known to bind to certain regions of core nucleosomes to mediate displacement of histones and participate in the regulation of specific genes by reducing intrinsic bends and increasing the affinity of transcription factors. Binding of CarD to the promoter could result in a conformational DNA change that facilitates binding of a signal specific transcription factor. Alternatively it could interact with signal dependent factors to form promoter specific complexes (Nicolas *et al.*, 1996).
1.6.9. The carE locus

This locus was identified through a Tn5 insertion, which abolishes carotenoid production and leads to the formation of pink colonies. Cloning and sequencing showed the locus encoded integration host factor (ihf) and subsequent Southern blots proved that *M. xanthus* had two copies of the gene. A further Tn5 induced mutation that blocked blue light carotenoid synthesis occurred at the promoter region of the *ihfA* gene, suggesting that the *ihfA* gene product functions as a positive element at an early stage of the regulatory cascade initiated by the light stimulus. It may participate directly in the activation of the *carQRS* promoter once the action of CarR has been blocked by light. The *ihfA* gene encodes the α subunit of IHF. This heterodimeric protein functions as an architectural factor in many processes including site-specific recombination, transcriptional regulation and replication (Nash, 1996). Its architectural function is due to its ability to induce a sharp bend in the DNA (Rice *et al.*, 1996). The participation of IHF has been reported in *Pseudomonas aeruginosa* in the activation of the *algD* promoter. The activation of the *algD* promoter depends on the formation of a high order loop structure (Baynham and Wozniak, 1996). Mutations in *ihfA* also block activation of the *crtI* promoter but this is indirect due to lack of induction of CarQ. In addition to hindering the light response, *ihfA* mutants are grossly impaired in cell motility. This is because IHF plays a critical role in the activity of σ54 dependent promoters so *ihfA* could be having an effect on expression from these genes (Moreno *et al.*, 2001).
1.6.10. The *carF* gene.

By screening the colour phenotypes of a collection of Tn5-*lac* insertion mutants, a new mutant devoid of carotenoid synthesis was isolated. The mutation was mapped to a new gene designated *carF* (Fontes et al., 2003). The *carF* deletion prevents the activation of the normally light inducible genes, without affecting the expression of regulatory genes. It does not affect the expression of the *ihfA* or *carD* genes. The CarF protein participates in the light-independent inactivation of CarR. A *carF* mutant behaves as a “blind” mutant being unable to perceive a light signal that usually leads to the inactivation of CarR. How CarF participates in the inactivation of CarR remains an open question though. Two mechanisms have been proposed, the CarF protein could be a primary photoreceptor being directly responsible for the reception of the light signal. This could cause a change in CarF that could then be conveyed to CarR. Alternatively CarF could be a signal transducer in the pathway from an as yet unidentified photoreceptor (Fontes et al., 2003). The CarF protein is homologous to the Kua proteins. These are found in *Mus musculus, Drosophila melanogaster, Caenorhabditis elegans* and *Arabidopsis thaliana* (Thomson et al., 2000). The Kua proteins are ubiquitinators, these target proteins for degradation in eukaryotes but are absent from prokaryotes.
1.6.11. Current model

The model to date is depicted in figure 1.8. In the dark the transmembrane protein CarR, binds to CarQ and holds it in an inactive state, while the CarA protein acts as a repressor of the *crtEBDC* operon. When the cells are exposed to light, PPIX is excited to a triplet energy state making it highly unstable. To regain its stability it transfers its energy to oxygen. This generates singlet oxygen which is incredibly reactive and very dangerous to the cell, as it can cause immense intracellular damage. The presence of singlet oxygen leads to the inactivation of CarR through the mediation of CarF although how is not yet clear. The loss of active CarR leads to the release of CarQ which initiates transcription at the *carQRS* and *crtl* loci leading to the generation of CarS. This then binds to the CarA protein and prevents it repressing the *crtEDBC* operon. Carotenoid tailoring enzymes are generated which together with phytoene dehydrogenase from the *crtl* gene leads to the production of carotenoids. The carotenoids produced quench the singlet oxygen and dissipate the energy harmlessly as heat, CarR levels increase as a result and CarQ is sequestered back to the membrane, a nice example of negative feedback.
Figure 1-8: Light induction of gene expression in *M. xanthus* – the current model

Events subsequent to illumination are depicted -detailed in the text.
1.7. Aims

This study aims to continue undertaking a fine structural analysis of the light-inducible promoter $P_{\text{carQRS}}$ which is the key operon in light-induction of the carotenoid regulon. The promoter has already been shown to be very large suggesting a complex regulatory regime. The initial aim was to continue studying previous mutations generated in $P_{\text{carQRS}}$ chapter two and three. A further more precise mutational analysis was also undertaken to clarify individual bases involved in -10 and -35 recognition by the sigma factor CarQ (chapter eight). The second aim was to investigate whether the $crtl$ gene was subject to auto regulation (chapter four). Previously identified genes downstream of the $crtl$ gene were assessed to investigate their roles in carotenogenesis (chapter five).
2. Molecular Analysis of the carQRS promoter

2.1. Introduction

2.1.1. Initiation of Transcription

Transcription of DNA into RNA and its subsequent translation are fundamental biological processes. In many species of bacteria, the number of genes is in excess of the number of functional RNA polymerase holoenzyme molecules (RNAP’s). Thus the distribution of RNAP’s between the different promoters must be strictly controlled, yet adaptable to changes. Within the cell RNA polymerase exists in two forms. The core polymerase is responsible for elongation during RNA synthesis and is comprised of five subunits ($\alpha_2\beta\beta'\Omega$). The initiating holoenzyme includes the relevant sigma factor ($\alpha_2\beta\beta'\Omega\sigma$) essential for promoter recognition (Burgess et al., 1969). The process of transcription is cyclic being subdivided into specific steps, pre-initiation, initiation, elongation, pausing and termination (Figure 2.1).

The RNA polymerase alpha subunit is 36.5KDa and is encoded by rpoA with two copies, in each of the core and holoenzyme. These form a dimmer, which is essential for RNA polymerase construction and directing DNA binding (Estrem et al., 1999). In E. coli the two genes which encode the largest subunits $\beta$ and $\beta'$ are transcribed from a single operon. The $\beta$ subunit is 150KDa and is encoded by rpoB. Together with $\beta'$ it forms the catalytic subunit (Heyduk et al., 1996). The $\beta$ subunit has also been implicated in rifampicin resistance (Severinou et al., 1998) and the stringent response (Ishihamo et al., 2000).
Figure 2-1: The transcription cycle (Finn et al., 2002).

E represents the core RNA polymerase, $\sigma$ represents the holoenzyme, which is the core associated with the relevant sigma factor. Pre-initiation involves the sigma factor binding to the core, the holoenzyme then locates the promoter known as promoter engagement. An initial binary DNA-protein complex is formed, termed transcription initiation. Following this the sigma factor is released leaving the core to carry out transcription elongation in a processive manner. After the gene has been transcribed, signals are conferred to the core RNA polymerase to release the mRNA and DNA this is termed termination.
When the holoenzyme binds to a promoter a closed complex is generated, with the DNA in a closed duplex state. Following isomerisation an open complex is formed. The DNA is unwound locally to expose a single stranded DNA template that can be transcribed. The DNA is orientated so as to allow interaction downstream with β' and upstream with β and β'. The complex of RNA polymerase, DNA template and new RNA transcript is called a transcription bubble. Then the complex reaches elongation mode. The RNA transcript forms a transient RNA-DNA hybrid helix with the template, which peels away from the DNA as transcription proceeds. The sigma factor remains attached until the nascent strand reaches 19-16 residues. This results in the release of the sigma factor, while the polymerase progresses until a termination signal is located.

2.1.2. Promoters of M. xanthus

Knowledge of promoters in M. xanthus has remained sketchy, being hindered by the presence of multiple sigma factors (Whitworth pers communication). It is important to know which sigma factor is responsible for expression of which promoter. Until then it will prove difficult to assign a consensus promoter sequence, for the housekeeping and alternative sigma factors. A summary of the sequences and proposed regions of interest of known M. xanthus promoters is shown (Figure 2.2).
δ^{70} like and constitutively expressed promoters

pkn5  AAATCTACGGACAGATACACGTTGCAACGTTGCAACG
pkn6  CAACATGACCCCGACGTACGGATTCGACAT
4403  GGTGATCATTAGAATAAGCCGTTGTTGATGTAACCGGCTTTTACC
frzZ  TTTCTAGGTCGGCTGCCGCAAAAGGTTGAGGCCTACCACGA
relA  CGTTCAGAAGTCGCCGAGAACACCCAGCCAGGGAGGACAGCA
sasS  GGTCACTCGAGGGACAGCCGTTGAGGCGCCACACTAAAAGTACGAC
vegA  TTTCTTTAGACAAAAACTTTTTGGAAGTTAGGTTAGGCA

Putative and proven SigECF-dependent promoters

CarQ-dependent-direct vs indirect

carQRS  CGAGCGCGGGAACACTTTTGCA GGTGCCCCTGATAGGAGTCG
crtI    GGTGCTCTTTGTAACGCTTCGCGCTTCCGACACCT

SigE-dependent

rpoEl  GCTAGGAATATGTTTTCTGCGCCTTGCCCTTGGCTTGGGCA
rpoE2  ACTCGGAGGGGATTTCCGCTCCTGCGGCTTGGGCA

unidentified promoters

4400  GGCGGAGGCGGCGAGGTGACATGCAGGCACACCCCGGTC
gufA   GCATCCCGCTCTGCTGGGCTTGGGCTTGGGCTTGGG

δ^{54} like promoters

carA  TTGGGGAAGGCGGAGGCCGCTTCCGCTTGGGCTTCCGCT
4521  GTGAGACGCGC TCTTTGCTTTGCTCAGGCCTCTTC
mbhA  GAATGGCAGCCACCTCT GCTTCGCGGTCCGCCGAGC

Figure 2-2: The -35 and -10 regions of promoters from M. xanthus

There are some obvious similarities at the -10 and -35 regions of some promoters compared to Sigma 70 dependent promoters of *E. coli*. The consensus promoter sequence for the *E. coli* housekeeping sigma factor is TTGACA...TATAAT. Several similar motifs can be seen in *M. xanthus*. For example the *relA*, *pkn6* and *crtEDBC* promoters have very similar hexamers at the -35 region. One base pair is different in each, with only *pkn6* showing similarity at the -10 region. The presence of hexamers which have a great degree of similarity to the *E. coli* consensus promoter sequence, suggests that there is at least one sigma factor in *M. xanthus*, which recognises a similar consensus to that recognised by Sigma 70 of *E. coli*. The gene encoding Sigma70 of *M. xanthus rpoD*, has been cloned and sequenced, with the gene product proving to be active *in vitro* (Inouye, 1990; Davies *et al.*, 1995; Biran and Kroos 1997).

The *carQRS* and *crtI* promoters are both require the sigma factor CarQ either directly or indirectly (McGowan *et al.*, 1993; Gorham *et al.*, 1996). This suggests that they may share some sequence identity. The expression of *crtI* is very different from that of *carQRS* though. *In vitro* transcription run off assays demonstrated that CarQ initiates transcription of the *carQRS* promoter but this could not be repeated for the *crtI* promoter (Browning *et al.*, 2003). This could be due to a missing factor required by the *crtI* promoter, but not the *carQRS* promoter. The *crtI* promoter is also regulated by carbon limitation but the *carQRS* promoter is not. Starving the cells of valuable nutrients leads to the activation of the *crtI* promoter. The repressive effect of carbon seen in the *crtI* promoter may be specific to the *crtI* promoter, or it could be a consequence of the nutritional state of the cell.
2.1.3. ECF sigma factor dependent promoters

Bacterial sigma factors belong to five large and apparently unrelated protein families. Group one sigma factors are essential proteins responsible for most transcription and include Sig70. Group two sigma factors are related to group one but are dispensable for growth. These include RpoS from *E. coli*. Group three was assigned in 1994 by Lonetto *et al* (1994), these sigma factors sometimes lack conserved region one and three. Group four are the extracytoplasmic function (ECF) sigma factors, small regulatory proteins that are quite divergent in sequence with regard to other sigma factors (Lonetto *et al*., 1994). The ECF group does not have region 1 or region 3. In the late 1980’s a biochemical approach led to the identification of two sigma factors in *S. coelicolor* and *E. coli*. In *E. coli* SigE was shown to account for transcription of the gene encoding the heat shock sigma factor Sig32 (Erickson and Gross, 1989). In *S. coelicolor* SigE was shown to direct transcription of the agarase encoding gene *dagA* (Buttner *et al*., 1988). Both sigma factors were shown to belong to a new subfamily of the sigma 70 family the ECF sigma factors. As a group they have several common features (Figure 2.3). Firstly they often recognise promoter elements with an AAC motif in the -35 region. They are often co-transcribed with a transmembrane anti-sigma factor with an extracytoplasmic sensory domain and an intracellular inhibitory domain. Finally they often control functions associated with some aspect of the cell surface or transport (Helmann, 2002).
Figure 2-3: Properties of a generic ECF sigma factor regulon (Helmann 2002).

A typical bacterial ECF sigma factor designated as sigma Z is co-transcribed with its downstream regulatory gene/anti-sigma factor rsiZ. Usually the anti-sigma factor is located in the membrane. Upon interaction with an extracytoplasmic signal sigma Z is released and is free to initiate transcription at Pz, and also activates transcription of other genes controlled by sigZ.
Since their discovery, hundreds of new members of the ECF family have been identified in a wide variety of Gram positive and negative bacteria. CarQ is a member of the ECF sigma factors. It shares many of the features mentioned above, including being co-transcribed with CarR, an anti-sigma factor.
2.1.4. Promoters of the *M. xanthus* carotenogenic regulon.

Evidence for the start site of the *crl1* promoter came from primer extension analysis (Fontes *et al.*, 1993). The start site of the *carQRS* promoter was mapped using transcription run off assays and primer extension analysis (McGowan *et al.*, 1993; Browning *et al.*, 2003). The *crl1* promoter showes little sequence similarity to the *carQRS* promoter at the -10 and -35 regions (Martinez-Argudo *et al.*, 1998). If the start site is displaced upstream by 12bp though, the sequence similarity with the *carQRS* promoter is increased. This promoter was referred to as *crl1 (+12)* (Browning, 1997). The minimum stretch required for *crl1* promoter activity was also determined (Martinez-Argudo *et al.*, 1998). Full expression required a stretch from between -54 and -25 to somewhere between +57 and +120. The reason for this large amount of downstream DNA remains unclear (Martinez-Argudo *et al.*, 1998).

The *carQRS* promoter region is exceptionally large for a bacterium. McGowan (1992) showed that the minimum stretch required for activity in the *carQRS* promoter extends from between positions -136 and -145, which includes the *gufA* promoter. The expression of the *carQRS* promoter is also dependent on the presence of CarD (Nicolas *et al.*, 1994). The *crl1* promoter also requires the presence of CarD (Martinez-Argudo *et al.*, 1998). This prompted a search for possible regions of homology to the binding sites for HMG1(Y) proteins in the *carQRS* promoter (Berry, 1998). A tandem repeat of TTTCC was located centred around positions -75 and -65. This repeat has not been found in the *crl1* promoter. Subsequent work proved that a CarD-GST fusion
would bind to the TTTCC repeat in the \textit{carQRS} promoter (Coyuels and Murillo, 1998). Yet various attempts have failed to show \textit{in vitro} binding of protein CarD to several DNA fragments that covered the entire length of the \textit{crtI} promoter (Martinez-Argudo \textit{et al.}, 1998). Despite the shared requirement for CarQ, the \textit{carQRS} and \textit{crtI} promoters share little similarity. Much confusion still remains about the \textit{crtI} promoter and its complex regulation.

2.2. Site directed mutations within the \textit{carQRS} promoter

Previous work generated fourteen site directed mutations spanning the entire length of the \textit{carQRS} promoter (Berry, 1998). Mutant sequences were designated \textit{mut1}-\textit{mut14} and were primarily engineered at sites so that the mutation introduced a \textit{KpnI} site. Each mutant promoter was fused upstream of a promoterless \textit{lacZ} gene allowing assessment of promoter activity. Constructs also contained a P1 \textit{inc} region, a kanamycin resistance determinant and an Mx8 \textit{attB} site (Figure 2.4). Construct pAEB600 carries the wild-type \textit{carQRS} promoter (Berry 1998).
<table>
<thead>
<tr>
<th>Mut14</th>
<th>Mut13</th>
<th>Mut12</th>
<th>Mut11</th>
<th>Mut10</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTA</td>
<td>GTA</td>
<td>TAC</td>
<td>GTAC</td>
<td>TAC</td>
</tr>
<tr>
<td>-155</td>
<td>GGTGCCCTCCGGGCCCCACACTAAAGGCCTCGCCCTCCAGGGCAGGACGGCAGGAGTCTTCGCGCCCTCCAGGGCAGGATGCT</td>
<td>+1 (gufA)</td>
<td>-10 (gufA)</td>
<td>-35 (gufA)</td>
</tr>
<tr>
<td>Mut9</td>
<td>Mut8</td>
<td>Mut7</td>
<td>Mut6</td>
<td>Mut5</td>
</tr>
<tr>
<td>GTAC</td>
<td>GGTA</td>
<td>GGTA</td>
<td>GTAC</td>
<td>GGT</td>
</tr>
<tr>
<td>-100</td>
<td>GCTGGCCGTTGCAACCCCGTGAACCTCCAGAGCTTTCCCTCACCG3ACCCCTTGAGAA</td>
<td>CarD BS</td>
<td>CarD BS</td>
<td></td>
</tr>
<tr>
<td>Mut4</td>
<td>Mut3</td>
<td>Mut2</td>
<td>Mut1</td>
<td></td>
</tr>
<tr>
<td>TAC</td>
<td>GGTA</td>
<td>TAC</td>
<td>TAC</td>
<td></td>
</tr>
<tr>
<td>-45</td>
<td>GCGCGAGCGGCCGGAAACACTTCGCAAGGGCCGAGTAGAGGAGGATGGCTGAGTGC</td>
<td>-35</td>
<td>-10</td>
<td>+1</td>
</tr>
</tbody>
</table>

**Figure 2-4: Site directed mutations within the carQRS promoter.**

The DNA sequence shown is the *EcoR*I fragment of pAEB120 containing the *carQRS* promoter sequence. Changes engineered in the sequence by site directed mutagenesis are shown above the wild-type sequence. The mut labels refer to individual promoter mutations carried on the pAEB series. For example mut1 is carried on pAEB601. All mutations introduce a novel *KpnI* restriction site (GGTACC). The base underlined at -144 is the transcriptional start site of the *gufA* gene. While the sequences underlined at positions -74 and -64 are the proposed *carD* binding sites.
2.3. Integration at the Mx8 \textit{attB} site

Mx8 is a general transducing phage of \textit{M. xanthus}. It is capable of a lysogenic lifestyle and was first isolated as a lysogen (Martin \textit{et al.}, 1978). Lysogeny arises due to a specialised integration system encoded within the phage. The Mx8 genome contains the \textit{attP} site which is a region of 29bp overlying a repeat of 11bp (Tojo \textit{et al.}, 1996). The \textit{attP} sequence is identical to a site on the \textit{M. xanthus} chromosome, the \textit{attB} (Mx8) site which is located 3.5Mb from the \textit{carQRS} locus (Chen \textit{et al.}, 1991). Site specific recombination across the \textit{att} sites allows the phage genome to recombine into the chromosome. As a result the \textit{intP} gene, which encodes an integrase, becomes truncated forming \textit{intX}. The gene product of \textit{intX} is devoid of any integrase activity.

Introduction of the pAEB600 series into the Mx8 \textit{attB} site, results in none of the genes in the carotenogenic regulon being disrupted. This allows activity of the promoters to be assessed in a wild-type background. Various levels of background activity have been noted in the Mx8 site, with different integrated genes, when compared to their activity in their natural location on the chromosome (McGowan, 1992). The activity of the \textit{carQRS} promoter is reduced at the \textit{attB} site possibly due to the distance between this site and the \textit{carQRS} operon. The activity from the promoter is still sufficient to assess changes in promoter activity due to the introduced mutations. This issue will be addressed further in chapter 8.
2.4. Checking the Mut constructs

Plasmids carrying mutated promoters were screened via PCR utilising primers 1 and 2. This allows the plasmid to be detected. Primer 1 anneals to a site within the carQRS promoter while primer 2 anneals in the opposite orientation within the lacZ gene. Products will only be obtained when the plasmid carries both in the correct orientation (Figure 2.5). Further confirmation for the presence of the mutation was obtained by restricting the 480bp PCR product with KpnI. The mutated promoters have a novel KpnI site. Cleavage confirmed that the copy of the carQRS promoter within the plasmid still possessed the engineered mutation (Figure 2.6).

PCR was also utilised to check that only one copy of the plasmid had been integrated. Primers multi one and multi two are complimentary to regions flanking the Mx8 attP site within the Mx8 attP region (Materials and Methods figure 7.2). Amplification between these primers should only occur in strains containing an intact copy of the attP site, i.e. in an E. coli strain carrying one of the pAEB6XX plasmids or in M. xanthus strains which contain tandem multiple insertions of the pAEB6XX plasmid in the Mx8 attB site. A positive control was built into the PCR reaction, because absence of a product does not mean that the target template sequence is not present. Primer multi three is complimentary to a site within the Mx8 attP site relative to attP and in the opposite orientation, thus amplification should occur between multi one and three in all strains (Figure 2.7). This is regardless of copy number and acts as an internal positive control (Whitworth, 1999).
Figure 2-5: Checking the pAEB6xx/mut constructs for plasmid orientation in the Mx8 attB site. PCR products were loaded on a 0.5% gel to check for the presence of the plasmid.

Lane 1: Marker. Lane 2: DK101. Lane 3: DK101::pAEB600 (wild-type). Lanes 4-12 putative constructs (DK101::pAEB608- pAEB614). Constructs DK101::pAEB608-614 were screened using primers 1 and 2. A 480bp product was only obtained when the plasmid carried both lacZ and pcarQRS in the correct orientation.
Figure 2-6: Screening for the presence of individual mutations within the carQRS promoter. PCR products obtained from using primers one and two were digested with KpnI to confirm the presence or absence of the mutation.

Lanes 1 and 4: Molecular markers. Lane 1: pAEB600 uncut. Lane 3: pAEB600 cut KpnI. Lane 5: pAEB610 uncut. Lane 6: pAEB610 cut KpnI. PCR products were digested with KpnI. The presence of a KpnI site between the carQRS promoter and the lacZ gene gives a 300bp and a 220bp product. Presence of an engineered site eliminates the 220bp fragment.
The PCR products expected from combinations of (MULTI) primers 1, 2 and 3 differ depending on location. In situation A the plasmid contains attP, B shows a single integrated plasmid at the Mx8 attB site, while C shows multiply integrated plasmids at the Mx8 attB site. All three give a product between primers 1 and 3, when using plasmid DNA as a template but a product is only formed between primers 1 and 2 when the plasmid carries attP (A) or has multiply inserted plasmids in the chromosome (C). Diagram is not to scale. (Whitworth 1999).
The PCR screen for multiple insertions was performed on all strains containing integrated plasmids. Amplification between primers multi one and two gave a product of 694bp, while amplification between primers multi one and three gave a product of 212bp, demonstrating the presence of integrated plasmids as tandem multiple insertions (Figure 2.8). This was important as beta-galactosidase assays had demonstrated significant increases in promoter activity compared to the wild-type for some mutants. It was therefore important to prove that the constructs were present in only a single copy.
Figure 2-8: Screening for multiple insertions at the Mx8 attB site. PCR products were run on a 1% gel to confirm the presence of the plasmid.

Lane 1 and lane 10: Marker. Lane 2: pAEB609 (plasmid DNA). Lane 3: DK101 (chromosomal DNA). Lanes 4-9: pAEB609-614 (Chromosomal DNA). Reactions were carried out using primers Multi 1, 2 and 3, to confirm that the plasmid had integrated. Amplification between multi primers 1 and 2 gave a product of 694bp, while amplification between primers multi 1 and 3 gave a product of 212bp. This proved that constructs 609-614 had multiple copies of the plasmid.
2.5. Mut 1-6 and mut 13-14

Previous work (Berry, 1998; Whitworth, 1999) had shown varying levels of \textit{carQRS} promoter activity (Table 2.1) depending on the location of the mutation in the \textit{carQRS} promoter. Mut1 proved to have no significant level of promoter activity. mut2 had a similar level of activity to the wild-type (Figure 2.9). Mut3 had a significantly elevated level of activity when compared to the wild-type. Mut4 had a decreased level of activity compared to the wild-type, while mut5 and 6 had slightly elevated levels of activity when compared to the wild-type (Whitworth, 1999). Both mut13 and mut14 demonstrated non light inducibility (Berry, 1998; Whitworth, 1999).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Peak specific activity during induction (units/min/mg protein)</th>
<th>Average specific activity in the dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Mut 1</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Mut 2</td>
<td>39</td>
<td>20</td>
</tr>
<tr>
<td>Mut 3</td>
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</tr>
<tr>
<td>Mut 14</td>
<td>16</td>
<td>13</td>
</tr>
</tbody>
</table>

\textit{Table 2-1: Comparison between average and peak specific activity in the light and dark. Experiments were done in triplicate average values are shown.}
Figure 2-9: Comparison between carQRS promoter activity in the Mut2 promoter (pAEB601), the Mut6 promoter (pAEB607) and the wild-type promoter (pAEB600) in the light.

Y-axis represents specific activity of beta-galactosidase in units/min/mg protein

X-axis represents time in hours
2.6. *In vivo* activity of the mutant promoters

The wild-type *carQRS* promoter is located on the plasmid pAEB600, designated Mut0. The plasmid was introduced into *M. xanthus*, DK101 using P1 mediated transduction. The resulting strain's production of β-galactosidase was assessed under illumination and without illumination (Figure 2.10). Assays were performed in triplicate and only consistent data are shown. New cultures were used for each new growth curve. In DK101::pDAH217 (promoterless *lacZ* gene fused to the light inducible *carQRS* promoter) the *carQRS* promoter is integrated at the *carQRS* locus instead of the Mx8 *attB* site. Activity in DK101::pDAH217 is greatly elevated in the light when compared to DK101::pAEB600 (400 units/min/mg protein compared to 35 units/min/mg protein respectively). This maybe due to the close proximity of the *carQRS* promoter in DK101::pDAH217 to the *carQRS* operon hence more readily available CarQ protein in the light. In DK101::pAEB600 there is some distance between the promoter and the *carQRS* operon, which is potentially responsible for the position effect seen.
Figure 2-10: Activity of the wild-type carQRS promoter at the attB site (DK101::pAEB600)

Y-axis represents specific activity of beta-galactosidase in units/min/mg protein.

X-axis represents time in hours
2.6.1. **Mut 7 exhibits no light induction.**

The mutation in Mut7/pAEB608 overlies the *carD* binding site at position -73 to -78, and abolishes light induction and background activity usually associated with the Mx8 *attB* site (Figure 2.11). Interestingly mut6/pAEB607 also overlies a *carD* binding site at position -63 to -67, yet this leads to a two fold enhancement in light induction (Whitworth, 1999). This increase in promoter activity when compared to the wild-type was thought to be caused by a reduction in predicted bending at the *carD* site (Berry, 1998). This could enhance CarD binding and promoter activity.
Figure 2-11: Activity of the Mut7 promoter (DK101::pAEB608) compared to the wild-type promoter pAEB600 (light and Dark).

Y-axis represents specific activity of Beta-galactosidase in units/min/mg protein

X-axis represents time in hours.
2.6.2. Mut8, Mut9, Mut10, Mut11 and Mut12 exhibit no light induction.

All mutations engineered between nucleotides -86 to -129 of the carQRS promoter (mut8-mut12), reduced promoter induction by a significant degree relative to the wild-type promoter. Mut8 is at position -86 to -89 and incorporates a 4bp change from $-89$CAAC$-86$-GGTA. The mutation is located upstream of the second CarD binding site (position -73 to -77) and between the -35 regions of the gufA and carQRS promoter. The level of promoter activity was greatly reduced compared to the wild-type carQRS promoter (Figure 2.12). A reduction in the background level at the Mx8 site was also noted from 15 units in the light in DK101::pSJM103 to 3 units in the light in mut 8. Mut9 is located at position -92 to -95 just upstream of mut8 with a 4bp change from $-95$CGTT$-92$-GTAC. Promoter activity in carQRS and Mx8 background activity are reduced significantly when compared to the wild-type (Figure 2.13).

Mut10 is at position -103 to -105 and has a 3bp alteration from $-105$ATG$-103$-TAC. Promoter activity was greatly reduced when compared to the wild-type (Figure 2.14). Mut11 is located at position -121 to -124 and has a 4bp change from $-124$CCCT$-121$-GTAC, this lies between the -10 and -35 region of the gufA promoter. Mut12 is located at -127 to -129 and has a 3bp change from $-129$TCC$-127$-CAT this overlies the -10 region of the gufA promoter. Both these result in loss of activity from the promoter when compared to the wild-type (Figures 2.15-2.16).
Figure 2-12: Activity of the Mut8 promoter (DK101::pAEB609) compared to the wild-type (pAEB600) in the light and dark.

Y-axis represents specific activity of beta-galactosidase in units/min/mgprotein.

X-axis represents time in hours.
Figure 2-13: Activity of the Mut9 promoter (DK101::pAEB610) compared to the wildtype (pAEB600) in the light and dark.

Y-axis represents specific activity of beta-galactosidase in units/min/mg protein.

X-axis represents time in hours.
Figure 2-14: Activity of the Mut10 promoter (DK101::pAEB611) compared to the wild-type pAEB600) in the light and dark.

Y-axis represents specific activity of beta-galactosidase in units/min/mg protein

X-axis time in hours
Figure 2-15: Activity of the Mut11 promoter (pAEB613) compared to the wild-type promoter (pAEB600) in the light and dark.

Y-axis specific activity of beta-galactosidase activity in units/min/mg protein.

X-axis time in hours.
Figure 2-16: Activity of the Mut12 promoter (DK101::pAEB614) compared to the wild-type promoter (pAEB600) in the light and dark.

Y-axis represents specific activity of beta-galactosidase in units/min/mg protein.

X-axis represents time in hours.
The complete loss of promoter activity seen in mutations mut10-12 suggests that these mutations all overlie crucial regions of the \textit{carQRS} promoter, and this could explain why they have reduced levels of activity compared to the wild-type (Table 2.2).

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Peak specific activity during induction</th>
<th>Average specific activity in the dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
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<td>10</td>
</tr>
<tr>
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<tr>
<td>Mut 8</td>
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<td>Mut 9</td>
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<td>Mut 10</td>
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</tr>
<tr>
<td>Mut 12</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

\textit{Table 2-2: Comparison between peak and average specific activity for the wild-type and the Mut constructs 7-12. Experiments were done in triplicate, average values are shown.}
2.7. Conclusions

Site directed mutagenesis has allowed the identification of important areas within the carQRS promoter. The mutation mut1 leads to non light inducibility and may be causing an alteration in the promoter which affects formation/activity of the RNA polymerase complex. Mut2 has no effect on promoter activity, this seems unsurprising as the mutation lies outside the area were promoter elements are believed to have a role in promoter activity. The mut3 mutation is constitutively active in M. xanthus, it remains constitutively active in a mutant strain deleted for CarQ. This strongly suggests that mut3 is expressed by another sigma factor (Whitworth, 1999). The promoter mutation mut4 lies at the 3'end of the highly conserved -35 promoter hexamer, and leads to a loss of light inducibility. This can be easily rationalised with CarQ no longer able to recognise the promoter. Both mut5 and 6 showed enhanced light induction, relative to the wild-type promoter. The presence of the mutation is postulated to cause a reduction in the angle of a predicted bend, intrinsic within the DNA of the promoter at the carD binding site. It could be that the mutations reduce the angle of curvature of an inhibitory bend within the promoter, allowing enhanced induction (Whitworth, 1999). The carD binding site at the carQRS promoter is a tandem repeat of the sequence TTTCC (Berry 1998). Regions which are A-T rich can introduce intrinsic curvature into DNA thus leading to an increase in transcription (Rao et al., 1994). By decreasing curvature, the sequence change in mut6 may be providing a carD binding site, which has a greater affinity for CarD than the wild-type.
Mut7 also overlies a carD binding site but unlike mut5 and mut6 it abolishes light induction and background activity. It could be that the carD binding site is the one predominantly recognised by CarD, and the subsequent mutation has rendered it inactive. This suggests that the two carD binding sites could have different functions within the promoter. Yet both sites are on the same side of the helix, making it difficult to envisage why mut7 would be favoured over mut6. It could be due to sequence surrounding the CarD binding site, which makes the CarD binding site at mut7 more favourably recognised over the site at mut6.

Mut8-mut14 are located at what appears to be a critical region in the promoter. The loss of promoter activity demonstrated by these mutants implies that the region between nucleotides -86 and -151 is critical for activity. The physical basis for this importance remains unclear since the DNA region in question is unusually far upstream. The loss of light inducibility seen in these promoters could be due to two possibilities. The reduction in activity could be due to disruption of the gunA promoter. The activity of the carQRS promoter should remain unaffected though, yet it is being affected suggesting a possible link between these two promoters, a topological dependence. This would mean that mutations in the gunA promoter would manifest themselves as loss of function carQRS promoter mutations. Alternatively the upstream region may include the binding site for one or more required transcription factors. Both theories are plausible and further analysis would provide further clarification.
The initial step in understanding the *carQRS* promoter has been taken with a gross mutational study, which has identified regions of function within the promoter (Figure 2.15). A further refined single base pair mutagenesis approach may help to illustrate which elements of the promoter dictate the various features of *carQRS* expression.

Red = No activity  
Blue = Wild-type activity  
Yellow = Very active  
Green = Slightly elevated

**Figure 2-17: Percentage induction of 6bp changes through the *carQRS* promoter.**

Mut1 shows no activity, mut2 has a similar level of activity to the wild-type. Mut3 has a highly elevated level of activity, while mut4 shows no light inducibility. Mut5 and mut6 have a slightly elevated level of activity. Mut7 also overlies a CarD binding site, yet shows reduced levels of activity. Mut8-14 all show no promoter activity, and led to the identification of a critical region.
3. Further characterisation of the carQRS promoter and the gufA promoter.

3.1. Introduction.

Previous work has shown that expression of the carQRS promoter requires the presence of the whole of the promoter of the upstream gene gufA (McGowan, 1993). It is possible that transcription of the gufA gene is required for transcription of the carQRS promoter. A possible role for gufA in carotenogenesis is discounted since a gufA knockout still retained its ability to generate carotenoids (McGowan, 1993). Another interesting observation is that expression from the gufA promoter is constitutive and does not alter its expression significantly upon illumination (McGowan, 1993). All carQRS promoter region mutations upstream of the CarD binding sites showed no significant light induction of transcriptional activity. It appears as though this region of the carQRS promoter forms a domain that is critical for activity of the carQRS promoter. The position of the critical domain correlates roughly to the location of the divergent promoter for the gufA gene. Two possible theories were put forward in the previous chapter to explain the critical region. One possibility was the existence of a transcription factor binding site. The large size of the carQRS promoter might reflect the presence of a binding site for a required transcription factor that could be operating through a DNA looping mechanism. The other possibility being that the two promoters were transcriptionally coupled through supercoiling. If two divergent promoters are found within a single topologically constrained domain, they can each stimulate transcription of the other promoter.
In order to discriminate fully between these two models (transcription factor binding vs transcriptional coupling) a series of mutant promoters were constructed, which carried deletions of 6bp stretches. Since there are approximately 11bp per helical turn of paired double-stranded DNA, removal of 6bp results in DNA upstream of the deletion being rotated about 180° relative to DNA downstream of the deletion. So if there is a transcription factor binding site upstream of the deletion there will be no activity from the carQRS promoter. If supercoiling links the two promoters then the activity from the carQRS promoter will be retained. The effect of each of the 6bp deletions on the activity of both carQRS and gufA promoters was assessed in vivo utilising beta-galactosidase assays. This would elucidate whether the expression of the carQRS promoter was linked to expression from the gufA promoter or simply due to the presence of a transcription factor binding site.
3.2. *In vitro* oligonucleotide directed mutagenesis of the *carQRS* promoter and the *gufA* promoter.

The plasmid pAEB120 contains a wild-type copy the *carQRS* promoter. This was used as a template for mutagenesis and had four separate 6bp deletions introduced (Figure 3.1). Deletion Δ*guf* was situated between the -10 and -35 regions of the *gufA* promoter, whilst Δ*car* was between the -10 and -35 regions of the *carQRS* promoter. The deletion in promoter Δ*mid* was located in the *carQRS* promoter region between the -35 region and the CarD binding sites, whilst that of the promoter Δ*int* was placed between the CarD binding site and the -35 region of the *gufA* promoter (Figure 3.1). Mutagenesis was carried out utilising PCR. Primers were designed to contain the desired mutation and anneal to the same sequence on opposite strands of the plasmid. Amplification around the plasmid pAEB120 led to the incorporation of the relevant 6bp deletions. Temperature cycling allowed the denaturation of the plasmid and subsequent annealing of the relevant primers, further extension and incorporation of the mutagenic primers resulted in nicked circular strands of DNA (Figure 3.2). The parental methylated non-mutated plasmid was removed by digestion with *DpnI* and used to transform MC1061. Transformants were selected by blue white screening and selective growth on ampicillin.

Transformants were screened using PCR with primers Δ*mid*, Δ*car*, Δ*int* and Δ*guf* to generate a product of 200bp which signified the presence of the *carQRS* promoter. The 200bp *carQRS* promoter fragment was excised, cleaned and sequenced to confirm the presence of the 6bp knockouts.
The DNA sequence shown is the EcoRI fragment of pAEB120 containing the carQRS promoter sequence. Changes in the sequence engineered by site directed mutagenesis are underlined and in bold on the wild-type sequence. Δguf represents a 6bp knockout between the -10 and -35 regions of the gufA promoter. Δint represents a 6bp knockout between the CarD binding sites and the -35 region of the gufA promoter. Δmid represents a 6bp deletion between the CarD binding sites and the -35 region of the carQRS promoter and Δcar represents a 6bp mutation between the -10 and -35 regions of the carQRS promoter. The -10 and -35 regions are shown for both the carQRS promoter and the gufA promoter.

The base underlined at position -144 is the transcriptional start site for the gufA gene.
The plasmid pAEB120 was used for amplification. Primers were designed to incorporate 6bp knockouts and anneal to opposite strands of the vector. The product is digested with \textit{DpnI} to remove methylated parental DNA. The plasmid is then transferred to \textit{E. coli} via transformation.
3.3. Construction of the pSJB100 plasmid series.

The mutated promoters were excised from pAEB120 using EcoR1 and inserted into the EcoRI site on the plasmid pAEB130. As a result plasmids were obtained with insertions in either orientation, allowing \textit{in vivo} assays of both \textit{carQRS} and \textit{gufA} promoter activities for the mutant promoter regions (Figure 3.3).

3.3.1. Checking the pSJB100 constructs.

Plasmids carrying the wild-type and mutant promoters were checked by PCR using PCR primers one and two (Table 8.9 Materials and Methods). Primer one anneals to a site within the \textit{carQRS} promoter while primer two anneals in the opposite direction within the \textit{lacZ} gene. A product of 480bp signified the presence of both the \textit{lacZ} gene and the \textit{carQRS} promoter in the correct orientation (Figure 3.4). Those promoter constructs present in the reverse orientation were screened by PCR using primer two and a reverse primer used in the original mutagenesis \textit{Δmid}, this anneals in the opposite orientation within the sequence (Figure 3.4). All the 6bp knockouts were screened in this way to confirm the presence and orientation of the mutations.
The *carQRS* promoter is flanked by *EcoRl* sites in pAEB120. It was digested with *EcoRl* and cloned into pAEB130. The presence of the *EcoRl* sites made selective orientation impossible meaning the *carQRS* promoter could be in either orientation in pAEB130. This was advantageous as it allowed both *carQRS* and *gufA* promoter activity to be assessed.
Figure 3-4: Checking orientation of the carQRS promoter in potential pAEB130 clones.

Lanes 1 and 11: Molecular marker. Lanes 2-10 PCR products derived from potential clones carrying the carQRS promoter in the correct orientation. Lanes 12-19: PCR products derived from potential clones carrying the carQRS promoter in the reverse orientation. Primer 1(pcarQRS) and 2(lacZ) were used to detect a 500bp product demonstrating the correct orientation of the carQRS promoter. Primers 2 and Δmid (6bp deletion- reverse primer) were used to detect a 500bp product depicting promoters in the reverse orientation.
3.4. **In vivo activity of the mutant promoters**

Plasmid pSJBl00 carries the wild-type *carQRS* promoter and the wild-type *gufA* promoter. The plasmid was introduced into the Mx8 *attB* site in DK101. The resulting strain's beta-galactosidase activity was assayed in both the light and the dark. The wild-type *gufA* promoter showed constitutive activity (Figure 3.5) while the wild-type *carQRS* promoter was light-induced (Figure 3.6).

**3.4.1. Δmid, Δcar and Δguf exhibit no light induction in both the *carQRS* and *gufA* promoters**

Δ*mid* incorporates a 6bp deletion between the CarD binding sites and the *carQRS* promoter. This abolished activity of both the *carQRS* promoter and the *gufA* promoter (Figure 3.7 and 3.8). The mutation at Δ*car* introduced a 6bp deletion between the -10 and -35 region of the *carQRS* promoter. It was assumed that such a deletion would render the promoter inactive, since the precise separation of these promoter elements is typically critical for promoter function. The promoter was inactivated in the light as a result of the mutation, activity remained low in the dark (Figure 3.9). Interestingly *gufA* promoter activity was also abolished in the Δ*car* mutation (Figure 3.10). The Δ*guf* mutation introduced a 6bp deletion between the -10 and -35 regions of the *gufA* promoter both *gufA* promoter activity and *carQRS* promoter activity were abolished (Figure 3.11 and 3.12). The simplest explanation for the above data is that the *gufA* promoter requires an active *carQRS* promoter and vice versa.

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Figure 3-5: Activity of the wild-type gufA promoter in the attB site.

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
Figure 3-6: Activity of the wild-type carQRS promoter in the attB site.

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
Figure 3-7: Activity of the Δmid carQRS promoter in the attB site compared to the wild-type carQRS Promoter DK101::pSJB100.

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
Figure 3-8: Activity of the Δmid gufA promoter in the attB site compared to the wild-type gufA promoter DK101::pSJB101.

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
**Figure 3-9:** Activity of the Δcar carQRS promoter in the attB site compared to the wild-type carQRS promoter DK101::pSJB100.

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
Figure 3-10: Activity of the Δcar gufA promoter in the attB site compared to the wild-type gufA promoter (DK101::pSJB101).

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
Figure 3-11: Activity of the Δguf gufA promoter in the attB site compared to the wild-type gufA promoter DK101::pSJB101.

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
Figure 3-12: Activity of the Δguf carQRS promoter in the attB site compared to the wild-type carQRS promoter DK101::pSJB100.

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
3.4.2. Δint exhibits light induction

The Δint mutation has a 6bp deletion between the CarD binding sites and the gufA promoter. This change had no significant effect on carQRS or gufA promoter activity. Instead of abolishing activity as previously seen in the other mutations a wild-type level of activity was retained in both promoters (Figure 3.14 and 3.15). If a transcription factor binding site required for carQRS promoter activity existed, then the reorientation of the binding site relative to the promoter would be predicted to abolish promoter function.
Figure 3-13: Activity of the Δint gufA promoter in the attB site compared to the wild-type gufA promoter (DK101::pSJB101).

Y-axis is specific activity of beta-galactosidase in units/min/mg protein.

X-axis is time in hours.
Figure 3-14: Activity of the Δint carQRS promoter at the attB site compared to the wild-type carQRS promoter (DK101::pSJB100).

Y-axis is specific activity of beta-galactosidase in units/min/mg protein

X-axis is time in hours
3.5. Conclusion

There were two possibilities proposed for the reduction in activity from the carQRS promoter. These were the presence of a transcription factor binding site or transcriptional coupling. It was proposed that the large size of the carQRS promoter would allow for the presence of a binding site for a required transcription factor that operated through a DNA looping mechanism. In most cases of activation through DNA looping, the loop is stabilised by architectural DNA-binding proteins that induce or stabilise bends in the DNA double helix. Examples include integration host factor (Thompson and Landy, 1988) and members of the HMG family of proteins (Thomas, 2001). In M. xanthus both integration host factor (Moreno et al., 2001) and a HMG(I) protein, CarD (Nicolas et al., 1994) have been shown to be required for transcription of the carQRS promoter in vivo, though neither are required in vitro (Browning et al., 2003). While binding sites for CarD within the carQRS promoter have been proposed (Nicolas et al., 1996), a binding site for IhfA has yet to be identified.

If a promoter exists on a stretch of DNA that is topologically constrained ie it cannot relax its supercoils, transcription from the promoter results in the accumulation of positive supercoils in front of the transcription complex, and negative supercoils behind the transcription machinery. Increased levels of negative supercoiling favour promoter melting during transcription initiation, therefore if two divergent promoters are found within a single topologically constrained domain, they can each influence transcription of the other promoter. Different promoters respond individually to changes in supercoiling.
The Δmid, Δcar and Δguf mutations all abolished carQRS and gufA promoter activity in vivo. This implied transcriptional coupling between the two promoters. The Δint mutation had no significant effect on either carQRS promoter activity or gufA promoter activity in vivo. This suggested that it was transcriptional coupling and not the presence of a transcription binding site that was responsible for the link between the two promoters. Reorientation of the binding site relative to the promoter would have abolished promoter activity in both the carQRS and gufA promoters if this was a transcription factor binding site. Since the Δmid mutation abolished carQRS promoter activity yet the Δint mutation did not, the orientation of the nucleotide sequence between positions -50 and -83 appears to be vital for carQRS transcription. This does not explain though why Δmid abolishes activity but Δint does not as both are close to CarD binding sites.

The above results suggest a possible link to transcriptional coupling leading to the formation of the critical domain between the two promoters. Anchoring DNA stretches such that they are unable to rotate freely can generate topologically constrained domains. Plausible mechanisms for constraining DNA in a domain include the binding of DNA-binding proteins (Dove and Dorman, 1994), the presence of bends in the DNA (Nelson, 1999) or through transient attachments to the membrane due to coupled transcription and translation of a transmembrane protein (Chen et al., 1992). A plausible topological domain is formed between gufA and carQRS since both GufA and CarR are predicted to contain transmembrane helices (McGowan et al., 1993).
The twin domain model (Liu and Wang, 1987) demonstrated that closely spaced divergent superhelically sensitive promoters can affect the transcriptional activity of one another by transcriptionally induced negative DNA supercoiling generated in the divergent promoter region (Liu and Wang, 1987). This gene arrangement has been seen in many LysR-type-regulated operons in bacteria including the *ilvYC* operon of *E. coli* where a downstream promoter mutation in the *ilvY* promoter severely decreased expression from the *ilvC* promoter, proving the two promoters were transcriptionally coupled (Rhee *et al.*, 1999). There are nineteen ECF sigma factors in *Pseudomonas putid*. Thirteen of these are involved in iron acquisition and pathogenicity. Six of these are encoded on operons with divergent promoters, which could possibly be transcriptionally coupled (Martinez-Bueno *et al.*, 2002). As CarQ is an ECF sigma factor this could possibly suggest a common requirement for supercoiling between divergent promoters in expression from promoters transcribed by ECF sigma factors. Although every gene has a 50% chance of being divergently transcribed, perhaps therefore this observation is not that surprising yet it still may suggest something about the regulation of genes transcribed by ECF sigma factors.
4. The *crtI* gene

4.1. Introduction

The *crtI* gene encodes phytoene dehydrogenase. This enzyme is responsible for the conversion of phytoene to lycopene via phytofluene, ζ-carotene and neurosporene. Lycopene is the initial coloured precursor in the C40 pathway. Carotenogenic structural genes have been cloned from such bacterial and fungal genera as *Rhodobacter*, *Erwinia* and *Neurospora* (Biel and Marrs., 1985; Armstrong *et al.*, 1998; Nelson *et al.*, 1989; Misawa *et al.*, 1990; Schmidthauser *et al.*, 1990). The genes have also been detected in *Streptomyces coelicolor* A3(2) (Bentley *et al.*, 2002) and *Streptomyces avermitilis* (Ikeda *et al.*, 2003) where they are light-regulated. In *Streptomyces griseus* (Horinouchi, 2001) the *crt* genes are not expressed.

Previous work in Francisco Murillo’s group had demonstrated that the *crtI* gene was controlled by a light inducible promoter. Activity from the promoter remains low in the dark. However it is strongly stimulated by illumination once the cells have reached the stationary phase (Fontes *et al.*, 1993). Mutations in the *carR* gene lead to constitutive expression from the *crtI* promoter. In *carQ* knockouts activity was completely abolished (Fontes *et al.*, 1993, McGowan *et al.*, 1993). This suggested that CarQ was regulating *crtI* gene expression. However *in vitro* transcription run off assays failed to reveal a transcript from *crtI* when utilising purified CarQ and *E. coli* core RNA polymerase, in conditions that revealed a transcript from *carQRS* promoter (Browning *et al.*, 2003). A further regulatory gene linked to *crtI* expression,
is the general transcription factor CarD. The *carD* gene is unlinked to other *car* and *crt* genes. It is expressed in a light-independent manner, and is required independently for the light activation of the *carQRS* and *crtl* promoters and is also necessary for germination.

Previous work had shown that in a *crtl* knockout expression from the *carQRS* promoter increases, due to lack of carotenoid feedback inhibition (Whitworth, 1999). Yet no data were available for expression from the *crtl* promoter in a *crtl* knockout. There are two possibilities; *crtl* expression in the *crtl* mutant could be greater than in the wild-type due to absence of carotenoid feedback. The absence of carotenoids should mean that any new CarR being generated from the *carQRS* operon should continue to be destroyed indirectly or directly by singlet oxygen mediation. CarQ should not be sequestered to the membrane and cytoplasmic levels should increase, promoting expression from the *crtl* promoter. A similar situation occurs in a *crtEBDC* knockout. Alternatively there could be a decrease in expression from the promoter due to *crtl* positive feedback. A role for CarQ in *crtl* expression had already been demonstrated. To elucidate if *crtl* is subject to carotenoid feedback *crtl* promoter activity was assessed in a *crtl* mutant. A suitable construct was generated using a *lacZ* fusion to assess activity from the promoter. Homologous recombination with the wild-type gene should generate a knockout and a promoter probe.
4.2. Construction of a *crtI* promoter probe

A plasmid was constructed from a pMTL backbone with the tetracycline resistance genes from Tn10. This was designated pDEW200 (D. Whitworth, per communication). This is a 5kb plasmid in which the tetracycline resistance gene is flanked by *PstI* and *XhoI* sites. Initially a 1.2kb fragment from the *crtI* gene was to be cloned into this vector, to generate the plasmid pSJB020. The plasmid pMAR202 was designed to allow transfer from *E. coli* to *M. xanthus*. It is unable to replicate in the latter. Successful transductants should only arise through integrative homologous recombination. The plasmid pMAR202 was generated from pMAR200 and contains the entire *crtI* locus (Fontes *et al.*, 1993). A restriction digest of pMAR202 with *PstI* and *XhoI* yielded a 1.2kb C-terminal fragment. This was cloned into pre-digested pDEW200 to yield pSJB020 (Figure 4.1). Successful constructs were identified utilising digests with *PstI* and *XhoI* to release the 1.2Kb fragment.

The plasmid pMAR206 contains a promoterless *lacZ* fusion to the *crtI* gene (Fontes *et al.*, 1993). The plasmid pMAR206 was digested with *BglII* and a partial *HindIII* and the plasmid pSJB020 was digested with *BglII* and *HindIII*. The 2kb fragment generated from pSJB020 was ligated into pMAR206 (Figure 4.1). This should have generated a *crtI::lacZ::Tet^R::crtI* fusion pSJB026, after screening several incorrect constructs this attempt was abandoned due to time constraints.
A 1.2kb fragment was excised from pMAR202 and ligated into pDEW200 to generate pSJB020. The crt-L::TetR fusion was removed from pSJB020 and ligated into pMAR206. This failed to generate a crt-L::lacZ::TetR::crtl fusion.
4.3. The promoter probe pSJB200

To effectively measure activity from the *crtI* promoter in a *crtI* knockout, a *lacZ* fusion was still desirable. A new strategy was devised utilising pDEW200 and DK101::pMAR206. This strain of *M. xanthus* has a copy of pMAR206 and an intact *crtI* gene. Primers were designed to amplify an internal 1Kb fragment from a wild-type copy of the *crtI* gene in DK101. Primers *crtlintfor* and *crtlintrev* also contained engineered *Bg*II sites to allow the fragment to be ligated into pDEW200. This generated pSJB200, which contained a *crtI*:Ter<sup>β</sup> fusion (Figure 4.2). Successful clones were screened using primers *crtlintfor* and *crtlintrev* to prove the 1kb fragment had been incorporated.

4.3.1. Integration of the plasmid pSJB200 into *M. xanthus*.

DK101::pMAR206 was transformed with pSJB200 utilising electroporation. Homologous recombination resulted in the generation of a *crtI* gene disruption. Successful electroporants were plated on tetracycline plates at 50μg/ml. Survivors were streaked to single colonies and screened via PCR, using primers designed to amplify the entire *crtI* gene. Primer *crtlf* was designed at the 5’ end of the gene while primer *crtlint* was designed at the 3’ end. Products of 1.5kb demonstrated that the gene remained intact (Figure 4.3). Those which did not have a 1.5kb band were assessed further. Potential successful transformants were subjected to
DK101 was transformed with pMAR206 to generate DK101::pMAR206. A 1kb internal PCR fragment from the wild-type *crtl* gene was amplified using *crtl*for and *crtl*intrev and cloned onto pDEW200 to generate pSJB200. This was electroporated into DK101::pMAR206 to generate DK101::pMAR206::pSJB200. This has a *crtl* gene knockout, PCR was used to confirm the construct utilising primers *crtlP*for and *crtl*intrev.

Figure 4-2: Generation of a *crtl* knockout.
Figure 4-3: Check for complete disruption of the crtl gene

1% agarose gel showing the products of a PCR reaction utilising primer crtlfor designed at the 5’end of the promoter and ctrlintrev designed at the 3’end of the crtl gene. Lane 1: Marker. Lane 5:DK101 (positive control). Lanes 2-4, 6-8, A-F: crtl-samples. The sample in lane D still has an intact copy of the crtl gene. Successful crtl knockouts are missing a 1.5Kb band.
illumination for 24 hours. True mutants were unable to synthesise carotenoids and lysed, those that retained their ability to generate carotenoids were discarded. The colonies, which failed to generate carotenoids were streaked to single colonies and used to assess promoter activity.

4.3.2. *In vivo* activity of the *crtI* mutant.

Previous work had demonstrated that after an initial lag period, induction of *crtI* occurs in the light with a maximum activity of 50 units/min/mg protein. This usually occurs after 6 hours and is known as phase I induction. After this initial activity there is a decrease in activity until about 18 hours post-innocation. Then a second phase of induction occurs which coincides with the entry into stationary phase, and is termed phase II induction (McGowan, 1992). This has a far greater magnitude than the first reaching 500 units/min/mg protein. In MC7 buffer lacking a carbon source phase I is absent, instead phase II induction is initiated prematurely (Fontes et al., 1993). Media replacement β-galactosidase assays were used to assess activity from the *crtI* promoter. After three hours cultures were centrifuged and pelleted, cells were resuspended in a buffer lacking a carbon source. Cultures were placed in both the light and the dark.

In DK101::pMAR206 in MC7 buffer induction was seen after 3 hours in the light, with maximal activity peaking at 430 units/min/mg protein (Figure 4.4). Activity was still retained at a low level in the dark at 30 units/min/mg protein. In DK101::pMAR206::pSJB200, there was an increase of greater than two fold in
promoter activity. To 1100 units/min/mg protein upon illumination, promoter activity was assessed three times to provide consistency in the data (Figure 4.4). Activity in the dark also increased to 50 units/min/mg protein. In DCY media phase I in DK101::pMAR206 peaked at just 50 units/min/mg protein in the light. In the dark it stayed relatively consistent at 15 units/min/mg protein.

In DK101::pMAR206::pSJB200 a similar level of activity was seen over a 12 hour period (Figure 4.5). Thus the differences seen in phase II induction were not obvious in Phase I induction. Two possible explanations can be envisaged for the increase in activity in DK101::pMAR206::pSJB200. Either *crl* negatively autoregulates itself or it is regulated by another factor. The data demonstrate that the *crl* gene is not subject to positive autoregulation.
Figure 4-4: Activity of the ctrl promoter in DK101::pMAR206 (WT) and DK101::pMAR206/crtI- with carbon limitation after 3 hours.

The Y-axis represents beta-galactosidase activity in units/min/mg protein.

The X-axis denotes time in hours.
Figure 4-5: Activity of the crtl promoter in DK101::pMAR206 (Wild-type) and DK101::pMAR206::pSJB200 in DCY media.

Y-axis represents specific activity of beta-galactosidase in units/min/mg of protein.

X-axis denotes time in hours.
The increased activity seen in DK101::pMAR206::pSJB200, could be due to loss of carotenoid negative feedback. As previously stated the absence of carotenoids will mean that any new CarR being generated from the carQRS operon will continue to be destroyed through singlet oxygen mediation. CarQ will not be sequestered to the membrane and cytoplasmic levels will increase, promoting expression from the crtl promoter. Alternatively the increase in activity could be due to crtl being subject to negative autoregulation. However if the crtl gene product has an auto repressor we would expect constitutive expression of crtl in the dark. Yet this is not the case. The observation that in a crtEBDC knockout a similar two-fold induction in crtl promoter activity was also noted (Whitworth, pers communication). This further supports the proposal that crtl is regulated by carotenoid negative feedback.
4.4. Conclusion

The above data strongly suggest that *crtI* is not subject to direct autoregulation. The *carS* and *carQ* deletions demonstrated the requirement for CarQ in *crtI* expression (McGowan *et al.*, 1993). While CarA was shown to have an indirect effect on expression from the *crtI* gene (Whitworth, 1999), and I have shown that *crtI* abolishes an indirect effect.

If CarQ binds to the *crtI* promoter, then this promoter must share some similarity to the *carQRS* promoter where CarQ also binds. Much uncertainty about the structure of the two promoters remains. A critical region called the downstream response element (DRE) was identified between +51 and +120 in the *crtI* promoter. This area proved significant if deleted, but remained active if displaced 27bp downstream of its normal position. Interestingly its activity was only slightly reduced when relocated upstream at position −59. This is also upstream of the RNA polymerase binding site. (Martinez-Argudo *et al.*, 1998). Sequence analysis of the promoter has shown that the *crtI* and *carQRS* promoters, share four of the five bases critical for *crtI* promoter activity at the −35 region (Martinez-Argudo *et al.*, 1998). In the −10 region three contiguous base pairs 5′-CGT-3′ are essential for activity. These are present at the *carQRS* site as well.
If the alignment of the *crtl* promoter is displaced downstream by 12bp a more extensive similarity between the −35 region of the two promoters can be identified (Browning, 1997). Although the −10 region still shows relatively little sequence similarity. Promoter sequence profiles for ECF (extracytoplasmic function) sigma factors demonstrate very clear similarities at the −35 region (Lonetto *et al.* 1994., and Hersberger *et al.*, 1995), which can be seen in the −35 region of the *carQRS* and *crtl* promoter when displaced by 12bp. This is different from the sequence found at non-ECF sigma factor dependent promoters. The −10 regions usually show little if any similarity. Presumably this allows sigma factor specific promoter discrimination.

The *carQ* mutations that render *crtl* inactive are strong evidence that CarQ regulates *crtl* gene expression. However the attempted *in vitro* transcription run off assays failed to demonstrate a direct requirement. These could have failed simply due to the absence of another factor required by CarQ to bind to the *crtl* promoter. CarD is a transcription binding factor which is linked to both the *crtl* promoter and the *carQRS* promoter. The protein CarD is absolutely required for activation of both the *crtl* and *carQRS* promoters *in vivo*. It binds to the *carQRS* promoter at A-T rich regions upstream of the −35 region (Nicolas *et al.*, 1996). It has been demonstrated that CarD is involved in CarQ-dependent transcription at both promoters. Yet no sequence critical for the activation of *crtl* is found outside of a DNA stretch extending from position −59 to position +120. Along this stretch a sequence similar to the CarD binding site at the *carQRS* promoter, could not be located. Furthermore no direct binding *in vitro* has been demonstrated for CarD and *crtl*, despite the entire length of the promoter being assessed (Argudo *et al.*, 1998).
This suggests two possibilities; either CarD and CarQ are required together to generate a transcript, or CarQ initiates the transcription of another sigma factor/transcription factor, which is responsible for regulating\textit{crtI} gene expression. This would explain the absence of a transcript in the \textit{in vitro} transcription run off assays.

Either model for \textit{crtI} regulation is plausible although a CarQ and CarD mediated regulation seems the more parsimonious explanation. This would mean that \textit{crtI} transcription mediated by CarQ would require CarQ, core RNA polymerase and CarD protein \textit{in vitro}. Yet transcription from the \textit{carQRS} promoter mediated by CarQ requires only CarQ protein and core RNA polymerase \textit{in vitro}. \textit{In vivo} CarD is required for transcription from the \textit{carQRS} promoter which explains why CarD binds to the \textit{carQRS} promoter. Yet no sites for CarD binding have been identified in the \textit{crtI} promoter. \textit{In vitro} transcription run off assays with CarD and CarQ would hopefully resolve this puzzle. Although a negative result would not prove that CarQ and CarD do not act together. It may be that the \textit{in vitro} transcription run off assays, do not account for structural conformations, which may be important in allowing the two to bind to the promoter. Future work with topologically constrained promoters could address the issue of how \textit{crtI} is regulated.
5. Analysis of the *crtI* region

5.1. Introduction

Sequence analysis of the region around the *crtI* gene identified three genes, one upstream of the *crtI* gene and two downstream (Whitworth, 1999). The smallest of the open reading frames identified was *olpA* encoding a small peptide of 100aa located downstream of the *crtI* gene. There is possibly translational coupling between the two as they overlap (Whitworth, 1999). The next open reading frame downstream of the *olpA* gene was denoted *gufB* (gene of unknown function B), this was found to share some sequence similarity with the PE PGRS (*Pro-Glu*, with polymorphic G-C repetitive sequences) genes of *Mycobacterium tuberculosis* (Whitworth, 1999). These PE PGRS proteins were identified in *M. tuberculosis* during the genome sequencing project (Cole *et al.*, 1998), and are very abundant and conserved proteins. Their role is thought to be as a source of antigenic variation. Any similar role in *M. xanthus* would seem somewhat surprising given that it is non-pathogenic. The *gufC* gene was identified upstream of *crtI*. Only the N-terminal portion of the open reading frame was available. The only sequence homology found was with *Saccharomyces cerevisiae*, and the vanadate resistance protein (Whitworth, 1999). This protein is responsible for the correct glycosylation and trafficking of proteins through the Golgi apparatus in *S. cerevisiae*. 
It is possible that all three genes could be involved in carotenogenesis due to their close proximity to the *crtl* gene. This is particularly true for the *olpA* and *gufB* genes, which are transcribed in the same direction as the *crtl* gene. The *gufC* gene presumably has its own promoter as it is transcribed in the opposite direction. It is possible that like the *gufA* gene, it may have no direct role in carotenogenesis, but a more indirect role through promoter supercoiling interactions.

5.2. The identification of an MCP homologue.

Availability of sequence data from Monsanto allowed assessment of sequence further downstream of the *gufB* gene. Initial analysis using Blast 2.0 and ORF finder (NCBI) identified a gene of 1953bp reading in the opposite orientation to *gufB* gene (Figure 5.1). This was initially designated *gufD* (gene unknown function). The start and stop codons were identified using Artemis. The *gufD* gene sequence was homologous to the *frzCD* gene of *M. xanthus*. This is a methyl accepting chemotaxis protein (MCP) involved in fruiting (Shi and Zusman, 1993). Frizzy mutants were initially identified in 1982 and showed tangled frizzy filaments under fruiting conditions (Zusman, 1982). The FrzCD protein is highly methylated during fruiting body formation (McBride and Zusman 1993). The *frz* genes were found to be required for controlling the frequency of reversal of cell movement. They encode proteins that are homologues to the major chemotaxis proteins of enteric bacteria (Blackhart and Zusman 1985; McBride *et al.*, 1989). The product of the *gufD* gene is therefore likely to be a methyl accepting chemotaxis protein.
It was hoped that gene knockouts would allow phenotypic analysis of the *olpA* gene the *gufB* gene and the *gufD* gene to define any possible role in carotenogenesis.

![Diagram of gene positions and transcription directions](image)

*Figure 5-1: Identification of an MCP homologue.*

Position of the open reading frames around *crtI* and the position of the newly identified *gufD* gene. The red arrow represents a known promoter. The thick black arrows represent putative promoters. The thin black arrows represent the direction of transcription. Diagram not to scale.
5.3. PCR-Targeted mutagenesis of *M. xanthus*.

A method was used to disrupt genes of interest, which had previously been demonstrated in *S. coelicolor* (Gust *et al.*, 2002). The plasmid pMAR202 contains the entire *crtI* region, and is unable to replicate autonomously in *M. xanthus*. This was introduced into BW25113 a strain of *E. coli* carrying the λ RED (*gam, bet, exo*) plasmid via electroporation. The λ RED plasmid functions to promote a greatly enhanced rate of recombination when using linear DNA. This was exploited in *E. coli* to engineer 40 different disruptions on the *E. coli* chromosome by replacing the wild-type sequences with a selectable marker (Datsenko and Wanner 2000). Successful BW25113:: λ RED:: pMAR202 electroporants were selected on chloramphenicol plates. A suitable resistance marker was selected to disrupt the genes. A kanamycin cassette could not be used due to the presence of kanamycin on the plasmid pMAR202. *M. xanthus* was streaked onto vancomycin, spectinomycin, streptomycin and apramycin plates. An apramycin cassette was selected after DK101 was shown to be sensitive to apramycin at 100µg/ml. The apramycin cassette was then used as a template for PCR. 39nt of homology to the gene of interest was incorporated either side of the apramycin cassette (Figure 5.2). A 1kb PCR product was generated comprising homology to the gene of interest, an apramycin cassette and the *frt* (recombination) regions. The PCR product was then electroporated into BW2113, which had the recombination plasmid and pMAR202. The λ RED plasmid recombination function was induced by the addition of L-arabinose and the PCR product was recombined into the plasmid pMAR202. The λ-RED plasmid is sensitive
The 5'-39nt sequence of the forward primer is from the coding strand of the gene of interest while the 5'39nt sequence of the reverse primer is from the complimentary strand. The 19nt and 20nt sequences match the right and left ends of the disruption cassette.

Figure 5-2: PCR primers for generating an in-frame deletion (Gust et al., 2002).
to temperature increments and was removed by increasing the temperature (Figure 5.3). Apramycin transformants were selected and the pMAR202 plasmid DNA was extracted using phenol/chloroform. Plasmid DNA was then digested with SsrI. This generated a band of 750bp (internal fragment within the disruption cassette), which verifies the presence of mutagenised plasmid DNA. The plasmid pMAR202 carrying the disruption cassette was then electroporated into DK101/DK1622. Successful electroporants were screened on apramycin and kanamycin plates. After successive plating, colonies failed to grow on kanamycin while retaining their ability to grow on apramycin, indicating a second crossover event had occurred. A final PCR reaction was used to verify the gene disruptants in DK101. This utilised primers designed 100-200bp upstream and downstream of the 39bp recombination region. A product of ~1kb proved that the cassette was present in DK101. These colonies were then used for phenotypic analysis.

5.4. Disruption of the *olpA* gene.

Despite several attempts this strategy failed to generate an *olpA* knockout. There are many plausible explanations for this. It could be that the *olpA* gene is essential and that a knockout is therefore not viable. The failure to generate an *olpA* knockout means that no firm conclusion can be drawn about the role of *olpA* in *M. xanthus*. 

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The plasmid pMAR202 was electroporated into the *E. coli* strain BW25113 which has a copy of the λ RED recombination plasmid pIJ790. The disruption cassette was amplified and electroporated into BW25113, a temperature was increase resulted in the loss of the λ RED recombination plasmid. The plasmid was then electroporated into *M. xanthus* to generate gene knockouts.
5.5. Phenotypic analysis of the *gufB* gene in DK101(WT).

Initially *gufB* mutants were screened for their ability to generate carotenoids. Both DK101 (wild-type) and UWM600 (*gufB*) were plated onto DCY plates containing ampicillin at 100μg/ml. One plate was illuminated for 48 hours while the other was wrapped in foil to prevent illumination. After 48 hours the plates were examined and the colonies were assessed. Both DK101 and UWM600 had generated carotenoids in the light and turned orange, while the ones in the dark remained yellow (Figure 5.4). This demonstrated that the *gufB* gene had no role in carotenogenesis. Many genes in the *M. xanthus* genome are involved in fruiting so it was decided to explore the possibility that the *gufB* gene may play a role in fruiting.

Cultures of DK101 and UWM600 were grown to late exponential phase in DCY media and then resuspended in TPM medium. This is a buffer which lacks a carbon source. Both DK101 and UWM600 were plated onto TPM medium and left for 72 hours. Initial stages of fruiting were monitored. After 24 hours DK101 showed initial signs of fruit formation (Figure 5.5), characterised by aggregation of cells and mound formation. When UWM600 was examined and compared to DK101 after 24 hours, in UWM600 cells had attempted to aggregate and aggregation was more advanced than the wild-type (Figure 5.5). This suggested that a mutation in the *gufB* gene had resulted in premature entry into development. After 72 hours the plates were examined again. DK101 had formed mature fruits all over the colony area (Figure 5.6).
Both DK101 and UWM600 generated carotenoids in the light.

Colonies from both strains remained yellow in the dark, demonstrating that the gufB gene is not required for carotenogenesis.
Figure 5-5: Developmental assays showing early fruit formation in DK101(WT) and UWM600 after 24hrs.

A- DK101 initial aggregation in response to starvation.

B- UWM600- aggregation is more rapid.
In UWM600 no mature fruits had been formed except on the very periphery of the colony (Figure 5.6 and Figure 5.7). This now suggested that increased numbers of cells at the periphery due to diffusion of the colony could restore the phenotype to wild-type and compensate for the mutation. The wild-type strain DK101 has a fruiting defect, which results in a slow progression to mature fruiting bodies (Hodgson pers communication). To assess the gufB mutation further it was compared to DK1622 which fruits normally.

Figure 5-6: Developmental assays showing late fruit formation in DK101(WT) and UWM600 in the colony centre.

A- DK101 mature fruit formation in the wild-type.

B- UWM600 no mature fruits are formed in the centre of the colony despite the initial rush to generate fruits, they remain immature even after 72 hours.
Figure 5-7: Developmental assays showing late fruit formation in DK101 (WT) and UWM600 at the periphery of the colony.

A- DK101 - late fruiting post 48hrs mature fruits are clearly visible.

B-UWM600 mature fruits are able to form at the colony edge perhaps due to larger cell numbers compensating for the mutation.

Immature fruits are still clearly visible though and predominate in the colony centre where cell numbers are relatively low. Broken lines represent the colony edge.
5.5.1. FLP mediated excision of the disruption cassette.

The apramycin disruption cassette is flanked by FRT sites (FLP recombination targets). Expression of the FLP-recombinase in *E. coli* removes the central part of the disruption cassette leaving behind an 81bp "scar" sequence which in the preferred reading frame lacks a stop codon. This allows the generation of non-polar unmarked in frame deletions. It was important to assess whether the *gufB* gene was responsible for the observed phenotype (the initial rushed entry into fruiting followed by a failure to generate mature fruits), as it could have been due to polar effects. The plasmid pMAR202 was electroporated into *E. coli* DH5α cells containing the temperature sensitive FLP recombinase plasmid BT340. The apramycin cassette was excised by thermal induction of the *flp* recombinase gene. This also resulted in loss of the plasmid BT340 due to its temperature sensitivity. Colonies were screened on apramycin and ampicillin plates, but this time, colonies were selected which grew on ampicillin but not apramycin. The plasmid was extracted as before and electroporated into *M. xanthus* DK1622. Colonies were screened on apramycin and ampicillin plates. Those that retained their ability to solely grow on ampicillin were screened further (UWM601).
5.5.2. Further analysis of the gufB gene in DK1622 (UWM601).

DK1622 and UWM601 were both plated onto TPM medium. Fruit formation was assessed after 24hrs and 72hrs. Initial signs of fruit formation could be seen in both, with slower aggregation in DK1622 compared with UWM601. In UWM601 early formation was seen as previously seen in UWM600. After 72 hours DK1622 had formed mature fruits, (Figure 5.8). In UWM601 fruits had only been formed on the periphery of the colony were numbers of cells increased, this was similar to the phenotype seen in UWM600 (Figure 5.8). The wild-type plasmid pMAR202 was electroporated back into UWM601 and restored the wild-type phenotype, (data not shown). This proved that the phenotype was due to the gufB knockout and not a secondary mutation elsewhere on the chromosome. If the gufB gene is involved in fruiting then it must be transcribed separately to the crtI gene, RT-PCR might confirm this hypothesis.
Figure 5-8: Developmental assays showing late fruit formation in DK1622 (WT) and UWM601

A- DK1622- mature fruits are formed.

B- UWM601- the phenotype remained the same as UWM600 no mature fruits were formed.
5.5.3. RT-PCR – Defining gufB as an independent gene.

To investigate the possibility that gufB was transcribed from its own promoter RT-PCR was utilised. Primers were designed at the start of the crtl and olpA genes and at the 3’end of the gufB gene and the olpA gene (Figure 5.9). Cultures of DK101 and UWM303 were grown for 72 hours in the light and the dark. RNA was extracted from both DK101 (WT) and UWM303 (Car-). RT-PCR was performed on all 4 samples with different primer combinations. Products were generated from DK101 between primers olpAfor and gufBrev and crtlfor and olpArev in the samples which had been illuminated. These products were very small though and not the product sizes expected. A product of 1.6kb should have been generated from crtlfor and olpArev but only a 500bp product was generated. While a product of 949bp should have been generated from primers olpAfor and gufBrev, instead products of 200bp were generated from samples of DK101 taken in the light and the dark. These small products could be due to primer mispriming. No product was found using primers crtlfor and gufBrev or with primers olpAfor and olpArev. The negative control was RNA with no reverse transcriptase enzyme added to the reaction mixture, meaning no DNA was present in the PCR reaction (Figure 5.10). The RT-PCR was therefore inconclusive and should be repeated in future work. Primer extension analysis was used to try and map the start site of the gufB promoter, but despite several attempts no product was generated, this was probably due to the quality of the RNA preparation.
Figure 5-9: Location of the RT-PCR primers.

Primer crtlf for was designed at the 5’end of the crtI gene, primer olpAf or was designed at the 5’end of the olpA gene. Primer olpArev was designed at the 3’end of the olpA gene and primer gufBrev was designed at the 3’end of the gufB gene. Amplification occurred between primers crtlf for and olpArev and olpAf or and gufBrev. No product was generated between crtlf for and gufBrev or olpAf or and olpArev, demonstrating that the gufB gene was linked to the olpA gene and not the crtI gene.
Figure 5-10: RT-PCR to determine whether the crtI gene and the gusB gene are co-transcribed.

Lane 1: DNA ladder. Lane 2: DK101 PCR product from primer \textit{crtI} for and primer \textit{olpA} rev in the light. Lane 3: PCR product from primer \textit{crtI} for and primer \textit{olpA} rev in the dark. Lane 4: PCR product from primer \textit{olpA} for and primer \textit{gusB} rev in the light. Lane 5: PCR product from primer \textit{olpA} for and primer \textit{gusB} rev in the dark. Lane 6: PCR product from primer \textit{crtI} for and primer \textit{gusB} rev in the light. Lane 7: PCR product from primer \textit{crtI} for and primer \textit{gusB} rev in the dark.
5.6. Phenotypic analysis of the MCP gene.

Being a \textit{frzCD} homologue and therefore a probable chemotaxis gene it seemed unlikely that the \textit{mcp} gene would have a role in carotenogenesis. This was still investigated though by plating the mutant UWM603 (MCP) and the wild-type (DK1622) onto DCY plates seeded with ampicillin at 100\textmu g/ml and illuminating one plate for 48 hours while the other was kept in the dark. Both strains placed in the light produced carotenoids suggesting that the MCP gene had no role in carotenogenesis. The plates left in the dark remained yellow as expected.

A possible role for the MCP gene in fruiting was investigated. Both DK1622 and UWM603 were grown to late exponential phase before being resuspended in TPM buffer and plated onto TPM plates. After 24 hours the plates were checked for early signs of fruiting similar to those seen in the \textit{gufB-} knockout UWM600/601. However both the wild-type (DK1622) and the mutant (UWM603) showed early signs of fruit formation (Figure 5.11). After 72 hours the mutant had formed deformed structures (Figure 5.12 and 5.13). Instead of mounds it had formed chain like structures. A-motility was still present in the mutant though suggesting a possible link to S-motility as S-motility allows cells to aggregate together to form mature fruits. The wild-type formed mature fruits as before (Figure 5.12).
Figure 5-11: Developmental assays showing early fruit formation in DK1622(WT) and UWM603

A- DK1622-fruits start to form after 24 hours with aggregation and initial mound formation.
B- UWM603- no signs of initial fruit formation could be seen.
Figure 5-12: Developmental assays showing late fruit formation in DK1622 (WT) and UWM603

A- UWM603- no mature fruits are formed just three dimensional chain like structures. A-motility is still viable though as protrusions can be seen from the colony edge.

B-DK1622- mature fruit formation.
Figure 5-13: Developmental assays showing late fruit formation in UWM603.

A- UWM603 forms long chain like structures after 72 hours. No other mature fruits are visible.

B- UWM603 generates a three dimensional structure.

These are unique to the mcp knockout and there are no examples in the literature of M. xanthus forming these structures.
Previously identified frizzy mutants formed tangled frizzy filaments under fruiting conditions (Zusman, 1982), but this was not the phenotype seen here. It seemed possible that the phenotype was due to contamination by another bacteria but streaking from the colonies on the TPM plates allowed recovery of just *M. xanthus* cells. To confirm that the phenotype was consistent, another MCP knockout was screened and generated a similar phenotype. Suggesting it was not a contaminant or a hair dropped in the agar for instance but a consequence of the MCP knockout. Complementation of the MCP knockout with the wild-type *gufD* gene restored the wild-type phenotype, further proving that the mutant phenotype was due to the *gufD* gene being removed and not the presence of a secondary mutation.
5.6.1. Chemotaxis assays

Chemotaxis has been shown to play a very important role in the social behaviour of *M. xanthus* (Shi *et al.*, 1993). Previous work had attempted to link chemotaxis and fruiting (Lev, 1954; Jennings, 1961; Fluegel, 1963; Shimkets *et al.*, 1979). Shi *et al* (1993) proved that *M. xanthus* could show chemotactic movement. As the *mcp* gene is a *frzCD* homologue and therefore a likely candidate MCP, chemotactic responses were investigated. Both wild-type (DK1622) and the MCP mutant (UWM603) were grown in CYE medium consisting of casitone, yeast extract and MOPS (Campos *et al.*, 1978). Both were then plated onto compartmentalised plates consisting of nalidixic acid (to repress growth) and agar. Testing substances were added to some compartments. These included yeast extract with casitone and 1.0% DMSO with 0.05% Isoamylalcohol. The plates were overlaid with sloppy agar to allow movement between the compartments. Cells were added and the plates were then incubated for 40 hours at 30°C and examined for signs of chemotaxis. The wild-type showed movement away from potentially toxic substances to more favourable medium on plates seeded with DMSO/isoamyralcohol and casitone. The mutant was unable to direct chemotactic movement away from the DMSO/isoamyl alcohol to the casitone. Controls were provided by mops only plates and casitone only plates. Both the wild-type and the mutant remained evenly distributed between the compartments, demonstrating that the mutant was clearly unable to respond to chemotactic gradients and therefore orchestrate a chemotactic response. Previous data had shown the importance of the *frz* genes in the chemotactic response in *M. xanthus* particularly with regards to the modification of the *frzCD* gene. The *mcp* gene is an *frzCD*
homologue, perhaps explaining why the cell is unable to function chemotactically when this gene is disrupted.

5.6.2. Motility assays

A-motility and S-motility show different selective advantages on different surfaces. Shi and Zusman (1993) demonstrated that A-motility allows cells to move better than S-motility on relatively firm and dry surfaces, while S-motility allows cells to move much better on relatively soft and wet surfaces. UWM603, DK1622, and DK101 were grown in DCY to late exponential phase. Cells were inoculated onto DCY medium with 1.5% agar. Both DK1622 and DK101 were able to swarm. DK101 has a defect in S motility and was therefore unable to swarm as well as DK1622. UWM603 was unable to swarm (Figure 5.14). Previous data (Shi and Zusman, 1993) proved that mutants defective in S-motility (A'S+) swarmed better than A-motility mutants (A'S'). Non motile mutants that lacked both motility systems (A'S') did not swarm and formed small colonies. UWM603 may be defective in A-motility as it failed to swarm. It is difficult to interpret whether the movement in UWM603 is due to colony expansion or S-motility. It may be defective in both forms of motility. This could be clarified by using an A-motility mutant (A'S') and a mutant defective in both (A'S').
DK101 and DK1622 are both able to swarm proving they are both motile.
UWM603 failed to swarm properly suggesting a possible defect in both A-motility and S-motility.
5.7. Defining a possible role for the gufC gene.

Previous annotation of the gufC gene had been limited due to sequence availability. The near completion of the \textit{M. xanthus} genome (TIGR) has made the analysis more informative. The gufC gene is transcribed in the opposite direction to the \textit{crlI} gene and may have no role in carotenogenesis. Like the gufA gene in the \textit{carQRS} operon any gufC promoter must lie close to the \textit{crlI} promoter. Its role in carotenogenesis could therefore be like that of the gufA promoter which is linked to the \textit{carQRS} promoter through supercoiling, making one promoter dependent on the other.

Sequence analysis using BLAST 2.0 demonstrated that the gufC gene encodes a sensor kinase containing a CHASE domain. These domains have only been discovered recently and are found in the extra-cellular portion of receptor-like proteins such as serine/threonine kinases and adenylyl cyclases. It is predicted to be a ligand binding domain (NCBI).

Most prokaryotic signal-transduction systems and a few eukaryotic pathways use phosphotransfer schemes involving two conserved components, a histidine protein kinase and a response regulator. Two component systems serve as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions. His-Asp phosphotransfer systems account for the majority of signalling pathways in bacteria but are rare in eukaryotes, where kinase cascades involving Ser/Thr and Tyr phosphorylation predominate. Both can function in eukaryotes and prokaryotes though, (Zhang, 1996; Loomis \textit{et al.}, 1997).
Most kinases are periplasmic membrane receptors. A Kyte-Doolittle plot for the gufC protein showed the presence of a transmembrane domain consistent with its role as a sensor kinase (Figure 5.15). Two component systems are very common in M. xanthus and are involved in a variety of functions from motility to fruiting. A gufC knockout will help to define its role in M. xanthus.

Figure 5-15: Kyte-Doolittle Hydropathy plot for the gufC gene

A hydropathy plot was used to detect potential transmembrane regions in the gufC protein. A score of 3.8 was obtained indicating that the protein is hydrophobic and therefore a potential membrane protein.
5.8. Conclusion

Initially it was postulated that the genes surrounding the *crtl* gene might have a role in carotenogenesis. Yet the genes around *crtl* seem to have different roles, predominantly linked to motility and possibly indirectly to fruiting. The role of the *olpA* gene still remains unclear. No real conclusions can be drawn about its speculative role until a mutant has been generated.

The phenotype seen in UWM600 and UWM601 is essentially identical and could be linked to a motility defect. A-motility remains unaffected in both strains suggesting that if it is a motility defect, then it might be an S-motility defect. This is because S-motility involves a social group movement, which requires cell sensing, signalling and aggregation, to form mounds of cells as precursors to the formation of mature fruits. Both UWM600 and UWM601 are unable to form mature fruits unless large numbers of cells are present to compensate for the mutation. The cells are capable of some aggregation and this premature aggregation leads to the formation of small mounds, which never reach maturity. The *csgA* gene encodes two proteins the CsgA protein and the C-factor protein. Previous work has shown that over expression of the CsgA proteins results in premature aggregation and sporulation and formation of small fruiting bodies (Kruse et al., 2001). However reduced synthesis of CsgA results in delayed aggregation and sporulation and the formation of large fruits. The *gufB* mutant shares some of the phenotypes associated with increased expression of CsgA i.e. it aggregates early. Yet aggregation in the *gufB* mutant never allows the formation
of fruiting bodies, suggesting gufB may have a role in motility and indirectly in fruiting. Time constraints prevented any further analysis of the gufB phenotype. Motility assays would confirm whether or not gufB had a role in S-motility and should be addressed in future work. Future work should also include varying the accumulation of the GufB protein as seen in the CsgA protein and investigating whether a gufB defect affects methylation of the frzCD gene.

The mutation in the mcp gene is perhaps the most interesting, the tangled filaments seen in other frizzy mutants is not characteristic of the mcp mutant. Yet their inability to undergo directed motility is characteristic of the mcp gene knockout. The formation of what appear to be long chain-like structures has not been previously documented in the literature and may be a unique phenotype of this mutation. It is probably due to an S-motility defect, although in the mcp gene knockout this cannot be compensated for by large cell numbers. Motility assays further hint at a role in motility but this needs clarifying further so that an S-motility defect can be distinguished from colony growth. The chemotactic response seen in the mcp gene knockout was typical for an mcp gene. The frzCD gene is modified by methylation, attractants cause methylation while repellents lead to demethylation. Hence methylation usually results from directed chemotactic movement (Shi et al., 1993). It has been hypothesised that the frz mutants are defective in sensing self-generating chemotactic signals that are required to attract cells into aggregation centres (Ward and Zusman 1997). It would be interesting to see if the phenotype seen in the MCP mutant could be rescued by increasing cell density. This would assess any role in signal generation rather than
signal transduction. The gufC gene has a possible role as a sensor kinase and could be defined further by gene knockouts which will demonstrate whether it has a role in carotenogenesis or fruiting.

The crtl region is certainly different from the other carotenogenic operons in M. xanthus like the carQRS and crtEDBC operons. In most other bacteria where a phytoene dehydrogenase gene is present including species of Erwinia, Flavobacterium, Rhodobacter, Streptomyces and Cyanobacteria, the crt genes are located together on one operon (Armstrong, 1997). Yet the crtl gene is in the middle of a motility operon in M. xanthus (Figure 5.16). This suggests that the crtl gene is a recent evolutionary addition to the genome. In all other crt operons crtl always proceeds crtB. In M. xanthus orf2 is directly upstream of crtB. There is evidence that in M. xanthus orf2 of the crtEBDC operon encodes an enzyme capable of converting phytoene into phytofluene (Murillo, F. J., pers communication), as transformation of E. coli with a plasmid carrying orf2, enabled the strain to produce copious amounts of phytofluene. Suggesting that orf2 was probably the original crtl gene responsible for the conversion of phytoene to lycopene. In a Δcrtl phytofluene still accumulates presumably due to the action of a gene product encoded by orf2 on the crtEBDC operon, suggesting orf2 may still have vestigial activity (Martinez-Laborda, et al., 1990). When M. xanthus acquired a second copy of crtl orf2 lost its PDH activity as 2 copies of the same gene were no longer required.
It is also interesting to note that a \textit{crtI} mutant had an affect on A-motility, which seemed somewhat surprising given its role in the production of phytoene dehydrogenase. The \textit{crtI} gene is also up-regulated in carbon starvation, a prerequisite for fruiting. Perhaps the newly acquired \textit{crtI} gene has a role in fruiting as well as carotenogenesis. Future work should address defining the role of the \textit{crtI} gene in fruiting and characterising the roles of the other genes on the \textit{crtI} operon more clearly to define their possible roles in fruiting and motility.

\textit{Figure 5-16: The \textit{crtI} region}

The \textit{crtI} gene has inserted into the middle of a motility operon. The \textit{che} genes are chemotaxis genes involved in detecting and mediating responses to environmental factors. The \textit{mcp} homologue is shown, next to hypo which is a hypothetical protein, \textit{gufC} is also present as HPK (Histidine protein kinase). HTH is a Helix-turn-Helix motif. SocD is a suppressor of \textit{csgA} involved in C-signalling. KefC is involved in potassium efflux. TodK is a Histidine protein kinase homologue and modulates C-signalling during fruiting body morphogenesis. DodR is similar to response regulators. Arrows denote direction of transcription. Diagram not to scale.
6. Discussion

6.1. The carQRS promoter

The carQRS promoter is a large complicated promoter with many regions of importance. The minimal stretch of DNA required for light-dependent transcription initiation of the carQRS operon consists of around 150bp of untranscribed DNA. Thectrl promoter is the only other promoter in M. xanthus known to be CarQ-dependent (Fontes et al., 1993). In contrast to the carQRS promoter, the ctrl promoter only requires 54bp of DNA upstream of the transcriptional start site (Martinez-Argudo et al., 1998). Mutations engineered to span the minimal promoter region showed a wide variety of changes to promoter activity. Only one mutation failed to affect promoter activity (Mut2) this overlies the transcription initiation site and thus would not be expected to be critical for promoter activity. Both Mut5 and Mut6 approximately doubled the induction of light-dependent transcription initiation in vivo. These changes lie upstream of the -35 recognition region and in the case of Mut6, overlap a proposed CarD binding site (Whitworth, 1999). Since the Mut7 promoter carries sequence changes overlapping the second CarD binding site, yet is completely inactive it appears as though the two CarD binding sites in the carQRS promoter have different roles. The positions of Mut5, Mut6, and Mut7 sequence changes relative to the -35 promoter element hint at interference with the normal binding of CarD.
When the -35 region was mutated (Mut4) transcriptional activity was abolished (Whitworth, 1999). This result was not surprising as sigma factor binding to the -35 and -10 promoter regions is required for transcription initiation at Sig70-dependent promoters and suggests that ECF sigma factor-promoter recognition is not radically different from that of the Sig70-like sigma factors. A particularly dramatic change in promoter activity was seen for the Mut3 promoter (Whitworth, 1999), where transcription was enhanced by several orders of magnitude and rendered light-independent. This increase in activity could be due to the recognition of the mutant promoter by a sigma factor other than CarQ (SigX), which is constitutively active during the LacZ assays (exponential growth phase). The transcription seen in the Mut3 promoter is further enhanced in a carQ deletion mutant suggesting that CarQ can still recognise the mutated Mut3 promoter in the wild-type and thus interferes with SigX-dependent transcription.

The mutant promoter Mut1 exhibited an abolition of light-induced transcription (Whitworth, 1999). Altered bases lie within the early transcribed region. It has been documented that base composition of the early transcribed region can have effects on transcriptional activity (Keene and Lüse, 1999; Buttner et al., 1987; Martinez-Argudo et al., 1986).
All mutations upstream of the CarD binding sites showed no significant light-induction of transcriptional activity. It appears as though this region of the carQRS promoter forms a domain that is critical for promoter activity. The position of the critical region correlates roughly to the location of the divergent promoter for the gufA gene. Such an important region is not seen for the crtI promoter, which nonetheless is associated with a presumed divergent promoter (Whitworth, unpublished results).

6.2. The gufA and carQRS promoters are transcriptionally coupled

The large size of the carQRS promoter might reflect the presence of a binding site for a required transcription factor that operates through a DNA looping mechanism. In M. xanthus both integration host factor (Moreno et al., 2001) and HMG(Y) protein, CarD (Nicolas et al., 1994), have been shown to be required for transcription of carQRS in vivo, though neither are required in vitro (Browning et al., 2003). Binding sites for CarD have been proposed in the carQRS promoter (Nicolas et al., 1996), yet the presence of a binding site for InfA has yet to be confirmed.
If a promoter exists on a stretch of DNA that is topologically constrained (cannot relax its supercoils) transcription from the promoter results in the accumulation of positive supercoils downstream of the transcription complex, and negative supercoils upstream of the transcription machinery. Therefore if two divergent promoters are found within a single topologically constrained domain, they each stimulate transcription of the other promoter (Dorman, 2002; Chen et al., 1992) A plausible topological domain is formed between *gufA* and *carQRS* since both GufA and CarR are predicted to contain transmembrane helices (McGowan et al., 1993). 6bp deletions were generated to discriminate between these two possibilities (transcription factor binding vs transcriptional coupling). Since there are approximately 11bp per helical turn of paired double-stranded DNA, removal of 6bp results in DNA upstream of the deletion being rotated around 180° relative to DNA downstream of the deletion. The Δ*int* promoter had 6bp between the CarD binding sites and the *gufA* promoter deleted. This change had no effect on *carQRS* or *gufA* promoter activity. If a transcription factor binding site required for *carQRS* promoter activity existed, upstream of the deleted bases, then the reorientation of the binding site relative to the promoter would abolish promoter function. This however was not observed which argues strongly in favour of transcriptional coupling between the *carQRS* and *gufA* promoters. Further support for this came from mutations Δ*guf* and Δ*car*. In these two promoters 6bp deletions were engineered between the -35 and -10 regions of the *gufA* and *carQRS* promoters respectively. It was found that in both deletion mutants, both the *gufA* and *carQRS* promoters had been inactivated by the sequence change. Thus it appears that it is impossible to inactivate one promoter without disabling the other promoter, again implying transcriptional coupling between the two promoters.
Transcriptional coupling was also observed for the Δmid mutant promoter, which had a 6bp deletion between the CarD binding sites and the -35 region of the carQRS promoter. The mutation inactivated both the carQRS and gufA promoters. Since the Δmid mutation abolished carQRS promoter activity, yet the Δint mutation did not, the orientation of the nucleotide sequence between positions -50 and -83 appears vital for carQRS transcription, suggesting a transcription factor-binding site. Indeed this region of the carQRS promoter does contain the two CarD binding sites.

6.3. The crtI region

A feedback loop, which regulates crtI expression, was confirmed by assessing crtI promoter activity in a crtI mutant strongly and suggested that crtI is not subject to direct autoregulation. The transcriptional activation of the crtI promoter still remains elusive, carQ mutations rendered crtI inactive but in vitro transcription run off assays failed to demonstrate a direct link between CarQ and the crtI promoter (Browning et al., 2003). This could have been due to a missing transcription factor like CarD which has been shown to be required by both the carQRS promoter and the crtI promoter, yet no binding sites have been identified in the crtI promoter (Martinez-Argudo et al., 1998). Both olpA and gufB were possible carotenoid genes yet a gufB mutant proved to have no role in carotenoid production, instead it looks as if its role is linked to motility. The complete genome sequence available from TIGR (http://www.tigr.org/) has completed the identification of genes around the crtI gene and none of them are carotenoid genes. They do however include chemotaxis (che) genes involved in sensing and co-ordinating responses to environmental signals and
other sensor kinases. This was unexpected given the arrangement of \textit{crt} clusters in other bacteria. In cyanobacteria for example all the \textit{crt} genes cluster together on one operon. It therefore seemed surprising to find \textit{crtl} inserted in the middle of a motility operon. This could be because \textit{crtl} is a recent addition to the genome, as \textit{crtl} usually precedes \textit{crtB}. In \textit{M. xanthus orf2} precedes \textit{crtB} suggesting that \textit{orf2} was formally \textit{crtl} and this lost its PDH activity following the acquisition of the new \textit{crtl} gene.

6.4. Future perspectives

Several aspects of the \textit{carQRS} promoter architecture remain to be defined, such as the nature of the binding site(s) for \textit{IhfA}. The relationship between the \textit{carQRS} and \textit{crtl} promoters also needs further clarification. Both promoters are \textit{CarQ} dependent yet they possess very different \textit{in vivo} and \textit{in vitro} behaviours. The individual base pair changes made in the -10 and -35 region of the \textit{carQRS} promoter need assessing. This will allow the importance of individual bases within the \textit{carQRS} promoter to be defined. The regulation of the \textit{crtl} promoter still needs to be investigated fully to assess the requirement for any other transcription factor. Finally future work should also address the motility mutants to define their roles more clearly in A-motility and S-motility.
7. Materials and Methods.

7.1. Bacterial strains and plasmids

7.1.1. Cultivation of E. coli

E. coli was cultivated at 30-37°C overnight in a shaker with/without the presence of selective antibiotics or streaked onto selective antibiotic Luria agar plates.

7.1.2. Storage of E. coli

For short term storage colonies were re-streaked every two weeks onto fresh selective plates. To retain the strains on a permanent basis the cells were cultivated overnight in 1ml Luria broth, then pelleted in an Eppendorf. The pellet was resuspended in 0.5ml of LB and 0.5ml glycerol and frozen at -80°C.

7.1.3. Media required for growth of E. coli

Luria Buitani (LB)  
1 litre water  
10g Tryptone  
5g Sodium chloride  
5g yeast

Luria Agar  
Add 15g of Agar/Litre
7.1.4. Cultivation of *M. xanthus*

*M. xanthus* strains were cultivated at $30^\circ$C for 2-3 days in a universal with the appropriate antibiotic selection. Gentle agitation was applied to aid growth or streaked onto DCY plates.

7.1.5. Storage of *M. xanthus*

*M. xanthus* strains were re-streaked onto fresh DCY plates with appropriate selection every 2-3 weeks. For long term storage cultures where grown over 2-3 days and 0.5ml was mixed with 0.5ml of DMSO. This was then frozen in the -80°C.

7.1.6. Media required for growth of *M. xanthus*

<table>
<thead>
<tr>
<th>DCY broth (1 litre)</th>
<th>DCY agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>20g casitone</td>
<td>15g Agar/litre</td>
</tr>
<tr>
<td>2g yeast extract</td>
<td>7.5g soft agar/litre</td>
</tr>
<tr>
<td>10mM Tris-HCL pH 8</td>
<td></td>
</tr>
<tr>
<td>8mM Magnesium sulphate</td>
<td></td>
</tr>
</tbody>
</table>
7.1.7. Antibiotics

Stock solutions were made and stored as described in Sambrook et al., (1989). Final working concentrations were generated and used according to the protocol.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100mg/ml</td>
</tr>
<tr>
<td>Apramycin</td>
<td>100mg/ml</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>12.5mg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50mg/ml</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>100mg/ml</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100mg/ml</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>12.5/20mg/ml</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>100mg/ml</td>
</tr>
</tbody>
</table>

7.1.8. Bacteriophage used

P1 Cl-100 Tn9 (Rosner, 1972).
7.1.9. Plasmids used

Detailed below are the plasmids used in this study. Names, descriptions and key features of each individual plasmid are provided.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAEB600</td>
<td>$\text{p}^\text{carQRS}$ (232bp BamHI/EcoRI fragment from pAEB120) $\text{lacZ, p1 inc}\Delta, \text{Mx8 attP, P15A ori, Km}^R$, (Berry, 1998).</td>
</tr>
<tr>
<td>pAEB601-616</td>
<td>Constructs identical to pAEB600 except that the copy of $\text{p}^\text{carQRS}$ had been mutated. pAEB601 carries the mutant promoter Alt1 (Berry, 1998).</td>
</tr>
<tr>
<td>pDAH274</td>
<td>$\text{lacZ, p1 inc}\Delta, \text{Ap}^R, \text{Km}^R, \text{P15A ori}$ (Hodgson, 1993).</td>
</tr>
<tr>
<td>pDEW200</td>
<td>Tet$^R$ from ColEl Tn5-132 into pMTL21 (Whitworth per communication)</td>
</tr>
<tr>
<td>pSJB200</td>
<td>pDEW200 with a 1kb $\text{crl}$ internal fragment.</td>
</tr>
<tr>
<td>pMAR202</td>
<td>$\text{crl}$ (20Kb KpnI/EcoRI fragment), Km$^R$, (Fontes et al., 1993).</td>
</tr>
<tr>
<td>pMAR206</td>
<td>$\text{p}^\text{crrl}$ and $\text{crl}$ (2.6Kb PstI fragment, $\text{lacZ, p1 inc}\Delta, \text{Ap}^R, \text{Km}^R$, P15A ori (Fontes et al., 1993).</td>
</tr>
<tr>
<td>pSJB700 series</td>
<td>Constructs identical to pSJB274 except they contain a copy of $\text{p}^\text{carQRS}$. This has been mutated.</td>
</tr>
<tr>
<td>pSJB274</td>
<td>$\text{lacZ, p1 inc}\Delta, \text{Ap}^R, \text{Km}^R, \text{P15A ori, orfX and orfY 1kb PCR product}$</td>
</tr>
<tr>
<td>pSJB700-724</td>
<td>$\text{lacZ, p1 inc}\Delta, \text{Ap}^R, \text{Km}^R, \text{P15A ori, orfX and orfY 1kb PCR product mutated p}^\text{carQRS}$ (Single base changes).</td>
</tr>
<tr>
<td>pIJ790</td>
<td>Datensko and Wanner, 2000 in Gust et al 2002</td>
</tr>
<tr>
<td>pBT340</td>
<td></td>
</tr>
</tbody>
</table>
7.1.10. *E. coli* strains used.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC1061</td>
<td>hsdR, mcrB, araD139, Δ(araABC-leu) 7679, galU, galK, rpsL, thi, ΔlacX74 (lacI).</td>
</tr>
<tr>
<td>DH5α</td>
<td>LacΔM15, ΔlacU169, recA1, endA1, hsdR17, SupE44, thi-l, gyrA, relA.</td>
</tr>
<tr>
<td>GM2929</td>
<td>F-ara-14, leuB6, thi-l, fhuA31, lacYI tsx-78, galK2, galT22, glnV44, hisG4, vpsL 136, (str&lt;sup&gt;R&lt;/sup&gt;), xyl-5MN-1 dam13::Tn9 (cam&lt;sup&gt;R&lt;/sup&gt;), dcm-6, mcrB1, hsdR2 (r&lt;sub&gt;Km&lt;sup&gt;i&lt;/sup&gt;&lt;/sub&gt;4&lt;sup&gt;+&lt;/sup&gt;) mcrA, recF143.</td>
</tr>
<tr>
<td>BW25113</td>
<td>Δ(araD-araB) S67, ΔlacZ4787, (:: rnb-4), lacZp-4000 (lacI&lt;sup&gt;K&lt;/sup&gt;), Aph5369(Am), rph-I, Δ(rhaD-rhaB) 568, hsdR514,</td>
</tr>
<tr>
<td>DH5α/BT340</td>
<td>Datsenko and Wanner 2000</td>
</tr>
<tr>
<td>XLI-Blue</td>
<td>F::Tn10 proA+B+, lacI, Δ(lacZ), m15/recA1, A1 gyrA96 (Na+) thi hsdR17 (r&lt;sub&gt;Km&lt;sup&gt;i&lt;/sup&gt;&lt;/sub&gt;4&lt;sup&gt;+&lt;/sup&gt;) glnV44, relAI, lac</td>
</tr>
</tbody>
</table>

7.1.11. *M. xanthus* strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Car genotype</th>
<th>Car phenotype</th>
<th>Derivation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK1622</td>
<td>Wild-type</td>
<td>Car+</td>
<td>Km&lt;sup&gt;3&lt;/sup&gt;A+S+ gliding</td>
<td>Kaiser (1979)</td>
</tr>
</tbody>
</table>
7.2. Basic techniques

7.2.1. Restriction endonuclease digestion of DNA

DNA was digested using the buffers and conditions supplied by the manufacturers (Gibco/Helena biosciences). Digestion was completed in a volume ranging between 10-50μl using 10-30 units of enzyme incubated at 37°C for 2-24 hours.

7.2.2. Reaction of alkaline phosphatase with DNA

DNA was 5'-dephosphorylated using calf intestine alkaline phosphatase using buffers supplied by the manufacturer in accordance with their instructions (Roche). The reaction was incubated for 1-2 hours at 37°C.

7.2.3. Reaction of T4 kinase with DNA

DNA was 5'phosphorylated by the addition of T4 DNA kinase and ATP conditions and buffers were supplied by the manufacturer (Gibco/Helena Biosciences).

7.2.4. Gel electrophoresis of DNA

Agarose gels were typically between 0.5-1% (w/v) and contained 1x TBE diluted from a 10x stock with 0.5μg/ml ethidium bromide. In order to visualise the gel and isolate individual bands a long-wave transilluminator was used to prevent the DNA
becoming mutagenised. A short-wave transilluminator was used to visualise and photograph the gel.

### 7.2.5. Materials needed for gel electrophoresis

<table>
<thead>
<tr>
<th>10X TBE</th>
<th>Loading buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>108g tris base</td>
<td>50% glycerol</td>
</tr>
<tr>
<td>55g boric acid</td>
<td>0.5% xylene cyanol FF</td>
</tr>
<tr>
<td>9.3g EDTA/litre</td>
<td>0.5% bromophenol blue</td>
</tr>
</tbody>
</table>

### 7.2.6. Transformation of *E. coli*

This was carried out according to the method described in Sambrook *et al.*, 1989.

### 7.2.7. Electroporation of *E. coli.*

This was carried out as detailed in Gust *et al.*, 2002 using a BioRad gene pulser. Exponential cells were pelleted and washed in cold 10% Glycerol (X3). The cell pellet was then resuspended in the remaining 100μl of 10% glycerol. 50μl of the cell suspension was then mixed with 100ng of plasmid DNA electroporation was carried out in a 0.2cm ice cold electroporation cuvette using a BioRad gene pulser II. This was set to 200Ω a capacitance of 25μF and 2.5kv. The time constant was between 4.5-4.9ms. Cells were flushed with 1ml of cold LB and incubated with vigorous shaking at 30°C before being spread onto selective media and incubated overnight at 37°C.
7.2.8. Transformation of *M. xanthus* by electroporation

Electroporation of *M. xanthus* was carried out as detailed in Hartzell 1995 using a BioRad gene pulser. Cells were grown to exponential phase and washed 3X in water before being electroporated. 40-100μl cells were added to 2-5μl of DNA in a 0.25cm electroporation cuvette, conditions for electroporation were set as follows, capacitance 25μF, voltage 0.65KV and a resistance of 400Ω. Time constants ranged from 0.5-0.9ms. Cells were flushed with 1ml of DCY and grown overnight in a total 3ml of DCY with the appropriate antibiotic. They were then overlaid with 2-3ml of soft DCY agar on selective plates colonies were visible after 3-4 days at 30°C.

7.2.9. Preparation of plasmid DNA from *E. coli*

Small scale preparation was carried out using the alkaline lysis QIAprep spin plasmid kit according to manufacturer’s instructions (Quiagen).

7.2.10. Large scale plasmid preparation

Cells were pelleted at 1300rpm in a microcentrifuge the pellet was resuspended in 100μl of solution I and 200μl of solution II. 150μl of solution III was added and the tubes were mixed 5x by inversion. The suspension was centrifuged at full speed (1300rpm/15.082g) in a microcentrifuge for 5 minutes at room temperature. The supernatant was then extracted using phenol/chloroform 25:1:1 (v/v) and vortexed for 2 minutes before being centrifuged for 5 minutes at 1300rpm/15.082g in a microcentrifuge. The upper phase was then mixed with 600μl of isopropanol. Tubes were left on ice for 10 minutes before being centrifuged for a further five minutes at
1300rpm/15.082g in a microcentrifuge minutes. The pellet was washed with 70% ethanol. Tubes were left open to allow any excess ethanol to evaporate. The pellet was then resuspended in 50μl of elution buffer.

7.2.11. Materials required for large scale preparation of DNA

Solution I
50mM Tris/HCL pH 8
10mM EDTA

Solution II
200mM Sodium hydroxide
1% SDS

Solution III
3M potassium acetate pH 5.5

phenol/chloroform

Elution buffer (Quiagen)

7.2.12. Caesium chloride gradient-Plasmid DNA extraction

10ml of an overnight culture of *E.coli* was sub-cultured into a one litre flask containing 500ml of LB and appropriate antibiotics. This was incubated overnight at 37°C with agitation. The cells were pelleted by centrifugation at 16,000rpm/22.623g in a HI spin 21 bench centrifuge for 30 minutes and then resuspended in 20mls of TES. This was followed by centrifugation at 10,000rpm/6.600g for 10 minutes in a HI spin 21 bench centrifuge. The cells were resuspended in 10ml of STE and frozen overnight. After defrosting, 1ml of lysozyme was added and the suspension was left on ice for 10 minutes. 16ml of triton-X was added and the solution was left on ice for a further 20 minutes until lysis occurred. After further centrifugation for 10 minutes at 10,000rpm/6.600g in a HI spin 21 bench centrifuge, the supernatant was decanted into a cylinder with 28.5g of caesium chloride. This was made up to 40ml with TSE.
and 1ml of 10mg/ml ethidium bromide. This was transferred to a VT150 tube. Tubes were balanced to within 0.01g and centrifuged at 45000rpm/234.00g in a Beckmann ultracentrifuge for between 14-24 hours. The plasmid DNA was collected by puncturing the tube with a needle and removing the upper band and then the lower band using a syringe. Ethidium bromide was removed using iso-amyl alcohol. An equal volume was added and the tubes were centrifuged at 4,500rpm/1.500g in a Wifug bench centrifuge. The lower layer was transferred to a clean tube and the procedure was repeated until both the lower and upper phase were clear. DNA was precipitated using six units of ethanol and two units of water. Tubes were left at -20°C for 2-4 days. The final pellet was resuspended in 50μl of water.

7.2.13. Solutions required for CsCl gradient

<table>
<thead>
<tr>
<th>STE</th>
<th>TES</th>
<th>Triton lysis mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>25% sucrose</td>
<td>50mM Tris/HCL</td>
<td>0.1% Triton X-100</td>
</tr>
<tr>
<td>50mM Tris-HCL</td>
<td>5mM EDTA</td>
<td>50mM Tris–HCL</td>
</tr>
<tr>
<td>5mM EDTA</td>
<td>50mM Sodium chloride</td>
<td>50mM EDTA</td>
</tr>
<tr>
<td>pH8</td>
<td>pH8</td>
<td>pH8.5</td>
</tr>
</tbody>
</table>

Lysozyme

10mg/ml Lysozyme

0.25M Tris-HCL pH 8

Elution Buffer

10mM Tris-HCL pH 8.5
7.2.14. Preparation of PI stock solution

Stock solutions of PI bacteriophage were prepared from single plaques of PI that had been grown on a lawn of *E. coli* (MC1061) on LC agar. Plaques were picked into TM buffer and plated onto LGC agar with MC1061 to generate a lawn of *E. coli* with confluently lysed PI plaques. Plates which exhibited confluent lysis were soaked with 6ml of TM buffer. The resulting PI stock was stored over chloroform.

Materials needed for preparation of PI stock solution

<table>
<thead>
<tr>
<th>LC medium</th>
<th>LGC medium</th>
<th>TM buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>As LB medium</td>
<td>As LC medium</td>
<td>10mM Tris-HCL pH8</td>
</tr>
<tr>
<td>5mM CaCl$_2$</td>
<td>2ml 50% Glucose</td>
<td>8mM MgSO$_4$</td>
</tr>
</tbody>
</table>

7.2.15. PI packing of plasmids for transduction of *M. xanthus*

The PI packaging of plasmids carried was carried out as described in Hodgson (1993).
7.3.  Physiological Studies

7.3.1.  Assays for the activity of beta-galactosidase in strains of *M. xanthus*

The protocol for assessing production of beta-galactosidase production in *M. xanthus* is detailed in Hodgson (1993).

7.3.2.  Media replacement during assays of beta-galactosidase activity

Assays which required the induction of cells into a medium lacking a carbon source were performed as described in Hodgson (1993) except that at t=3hrs the cultures were pelleted and split into four. Two were resuspended in 200ml DCY and two were resuspended in 200ml MC7 buffer after initial washing in 100ml of MC7 buffer. One of each was left in the dark and in the light. Sampling and enzyme activity resumed as normal.

7.3.3.  Materials required for media replacement beta-galactosidase assays

MC7 buffer

10mM MOPS
1mM CaCl₂
pH 7
7.3.4. Developmental assays

The assays for starvation induced fruiting body formation were performed as detailed in Yang and Kaplan (1997). A late exponential phase culture of *M. xanthus* was centrifuged at 4,500rpm in a Wifug bench centrifuge. Pelleted cells were resuspended in 1/10 volume of TPM. 20μl drops were then spotted onto TPM agar plates. These were incubated at 33°C in the dark. Mature fruiting bodies were generally formed after 72 hours.

TPM

10mM Tris-HCL pH 7.5
1mM KH$_2$PO$_4$
8mM MgSO$_4$

TPM agar
15g Agar

7.3.5. Chemotaxis Assays

Assays were performed as detailed in Shi *et al.* (1993). Compartmentalised Petri dishes were used to establish steep and stable chemical gradients. Petri dishes were filled with 0.3% agar (agar + water + nalidixic acid 100μg/ml), some compartments contained yeast and casitone (2mg/ml) and others contained iso-amyl alcohol/DMSO (0.05%/1.0% respectively). The plates were overlaid with 0.3% Mops buffer (10mM pH 7.6). Exponential phase cultures were spotted onto the borders between the compartments. The plates were left at 30°C for up to 20 hours. Movement of cells away from repellents or to attractants was measured using a ruler.
7.3.6. Motility Assays

*M. xanthus* cells were grown to exponential phase and then spotted onto CYE plates containing 0.3% and 0.5% agar as described in Shi and Zusmann (1993). The plates were incubated at 30°C for 20 hours. Swarming and hence motility was recorded by measuring the diameters of the swarming colonies.

7.3.7. Materials required for motility assays

CYE Agar

- 10mM Tris-HCL pH 7.5
- 1mM KH₂PO₄
- 8mM MgSO₄
- Agar at 0.3-1.5%


7.4. Molecular studies

7.4.1. Site directed mutagenesis of *E. coli*

A supercoiled dsDNA vector was selected (pAEB120 5kb) which has the *carQRS* promoter (Berry, 1998). Oligonucleotides were designed to incorporate single base pair changes and six base pair deletions. Primers were in pairs, complimentary to opposite strands of the vector. Temperature annealing was carried out as detailed in the table below using *Pwo*(Roche) which has a proof reading function to minimise the possibility of further mutagenesis. Incorporation of the primers generated a mutated plasmid. Restriction digests with *DpnI* (Stratagene/NEB) resulted in digestion of the parental DNA. The plasmid was then transferred to *E.coli* XL-Blue.

7.4.1.1. PCR conditions

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Time (secs)</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>35</td>
<td>1 minute</td>
<td>30</td>
</tr>
<tr>
<td>Elongation</td>
<td>68</td>
<td>4 minutes</td>
<td></td>
</tr>
</tbody>
</table>
### 7.4.1.2. Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCCGACTCTCTCTACGGGCGCTCCTGCAGAAG</td>
<td>Δ-10forI carQRS promoter pSJB707</td>
</tr>
<tr>
<td>CTTTCGCAGGCGCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revI carQRS promoter pSJB707</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGCCCCTGCAGAAAG</td>
<td>Δ-10forII carQRS promoter pSJB708</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revII carQRS promoter pSJB708</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-10forIII carQRS promoter pSJB709</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revIII carQRS promoter pSJB709</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-10forIV carQRS promoter pSJB710</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revIV carQRS promoter pSJB710</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-10forV carQRS promoter pSJB711</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revV carQRS promoter pSJB711</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-10forVI carQRS promoter pSJB711</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revVI carQRS promoter pSJB712</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-10forVII carQRS promoter pSJB712</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revVII carQRS promoter pSJB713</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-10forVIII carQRS promoter pSJB713</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revVIII carQRS promoter pSJB714</td>
</tr>
<tr>
<td>CCCGACTCTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-10forIX carQRS promoter pSJB714</td>
</tr>
<tr>
<td>CTTTCGCAGGCTGCCCCGTAGAGGGAGTCGGG</td>
<td>Δ-10revIX carQRS promoter pSJB715</td>
</tr>
<tr>
<td>CACGGACTCTCTACGGGCGACCTTGCGAAAG</td>
<td>Δ-35forI carQRS promoter pSJB715</td>
</tr>
<tr>
<td>CTTGGAGGAGCGCGCGCGCGAGAACACTTTGCGAGG</td>
<td>Δ-35revI carQRS promoter pSJB701</td>
</tr>
<tr>
<td>CTTGGAGGAGCGCGCGCGCGAGAACACTTTGCGAGG</td>
<td>Δ-35forII carQRS promoter pSJB702</td>
</tr>
<tr>
<td>Sequence</td>
<td>Promoter and Additional Information</td>
</tr>
<tr>
<td>--------------------------------------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>CCTCGCAAAAGTGTTCGGCGTGCTCGGCTTC</td>
<td>Δ-35revII carQRS promoter pSJB702</td>
</tr>
<tr>
<td>GAAGCGCGAGCGCCGAAACACTTTTCGCAAGG</td>
<td>Δ-35forIII carQRS promoter pSJB703</td>
</tr>
<tr>
<td>CCTCGCAAAAGTGGGCGCTCGGCTTT</td>
<td>Δ-35revIII carQRS promoter pSJB703</td>
</tr>
<tr>
<td>GAAGCGCGAGCGCAACAACTTTTCGCAAGG</td>
<td>Δ-35forIV carQRS promoter pSJB704</td>
</tr>
<tr>
<td>CCTCGCAAAAGTTGGCGCTCGGCTTT</td>
<td>Δ-35revIV carQRS promoter pSJB704</td>
</tr>
<tr>
<td>GAAGCGCGAGCGCAACACCTTTTCGCAAGTGG</td>
<td>Δ-35forV carQRS promoter pSJB705</td>
</tr>
<tr>
<td>CCACCTGCAGAAGTTGGCGCTCGGCTTC</td>
<td>Δ-35revV carQRS promoter pSJB705</td>
</tr>
<tr>
<td>GAAGCGCGAGCGCAAACTTTTCGCAAGTGG</td>
<td>Δ-35forVI carQRS promoter pSJB706</td>
</tr>
<tr>
<td>CCACCTGCAGAAGTGTTCGGCGTGCTCGGCTTC</td>
<td>Δ-35revVI carQRS promoter pSJB706</td>
</tr>
</tbody>
</table>
7.4.1.3. Site directed mutations in the *carQRS* promoter.

Red letters indicate bases which have changed or been added. Deleted bases are represented by a dash (-).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Sequence before mutagenesis</th>
<th>Sequence after mutagenesis</th>
<th>-10/-35 carQRS promoter</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSJB700</td>
<td>Wild-type</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>pSJB701</td>
<td>CCGGAA</td>
<td>.CGGAA</td>
<td>-35</td>
</tr>
<tr>
<td>pSJB702</td>
<td>CCGGAA</td>
<td>CGGGAA</td>
<td>-35</td>
</tr>
<tr>
<td>pSJB703</td>
<td>CCGGAA</td>
<td>CCCGAA</td>
<td>-35</td>
</tr>
<tr>
<td>pSJB704</td>
<td>CCGGAA</td>
<td>CCGCAA</td>
<td>-35</td>
</tr>
<tr>
<td>pSJB705</td>
<td>CCGGAA</td>
<td>CCCGAA</td>
<td>-35</td>
</tr>
<tr>
<td>pSJB706</td>
<td>CCGGAA</td>
<td>CCCGGAA</td>
<td>-35</td>
</tr>
<tr>
<td>pSJB707</td>
<td>CGTA</td>
<td>CATA</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB708</td>
<td>CGTAG</td>
<td>CGTAC</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB709</td>
<td>CCCGTA</td>
<td>GCCGTA</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB710</td>
<td>CCCGTAGA</td>
<td>CCGTAC</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB711</td>
<td>CGTA</td>
<td>GGTA</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB712</td>
<td>CGTA</td>
<td>CGTT</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB713</td>
<td>CTGAGA</td>
<td>CTGAA</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB714</td>
<td>CCCGTA</td>
<td>CACCGTA</td>
<td>-10</td>
</tr>
<tr>
<td>pSJB715</td>
<td>CGTA</td>
<td>CCGTA</td>
<td>-10</td>
</tr>
</tbody>
</table>
### 7.4.1.4. Primers for 6bp deletions

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position of mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>cactaagggctcaggagaggacggg</td>
<td>Δguffor (between -10 and -35 of the gufA promoter)</td>
</tr>
<tr>
<td>cccgtctgccctgcaggctcttagtg</td>
<td>Δgafrev (Between the -10 and -35 of the gufA promoter)</td>
</tr>
<tr>
<td>ctgctgctgctcgcttgactttccag</td>
<td>Δinffor (Between -35 of the gufA promoter and -35 of the carQRS promoter)</td>
</tr>
<tr>
<td>ctgggaagtcagcgaagggcagcag</td>
<td>Δinffrev (Between -35 of the gufA promoter and -35 of the carQRS promoter)</td>
</tr>
<tr>
<td>gctttctctacgcagaagggcagcagc</td>
<td>Δmidfor (Between the -35 of the gufA promoter and the -35 of the carQRS promoter)</td>
</tr>
<tr>
<td>gctgcgcgctctcaggttagaggaagc</td>
<td>Δmidrev (Between the -35 of the gufA promoter and the -35 of the carQRS promoter)</td>
</tr>
<tr>
<td>ccgggaacacaggtggccgagggagg</td>
<td>Δcarfor (Between the -10 and -35 regions of the carQRS promoter)</td>
</tr>
<tr>
<td>ccctactgggccccacttgctcttagg</td>
<td>Δcarev (Between the -10 and -35 regions of the carQRS promoter)</td>
</tr>
</tbody>
</table>

### 7.4.2. Large scale preparation of RNA from M. xanthus

Protocol used is detailed in Scanlan et al (1993). Cells were harvested during exponential growth by centrifugation at 8000 rpm/4.500g in a HI spin 21 centrifuge. The pellet was washed in 20ml of wash buffer and then resuspended in 1.6ml of RNA extraction buffer. The suspension was boiled in a microchef microwave on setting two for twenty seconds and then split into two Eppendorfs with 0.8ml of saturated phenol at pH 4.5. After mixing the cells were placed at 65°C for 5 minutes and then centrifuged in a HI spin 21 centrifuge at 8,000rpm/4.500g for 5 minutes.
The top aqueous phase was transferred to a fresh tube and extraction was carried out once with phenol and once with chloroform/iso-amyl alcohol (24:1:1 v/v). The RNA was then precipitated using ethanol and the pellet dissolved in 400μl of DNase buffer (10mM sodium acetate and 10mM magnesium chloride pH 5.6). 5μl of DNase I was added and the reaction was incubated for 1 hour at 37°C before a second enzyme addition of 5μl and a further incubation for 1 hour. The RNA was extracted again with phenol and chloroform/iso-amyl alcohol, the RNA was ethanol precipitated and resuspended in 50μl DMPC which contains 1 unit of RNA guard (Roche) to prevent RNA degradation.

7.4.3. Materials required for large scale RNA preparation from *M. xanthus*

<table>
<thead>
<tr>
<th>Wash buffer</th>
<th>RNA extraction buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05M EDTA</td>
<td>100mM Lithium chloride</td>
</tr>
<tr>
<td>0.12M Sodium Chloride</td>
<td>50mM Tris-HCL</td>
</tr>
<tr>
<td></td>
<td>30mM EDTA</td>
</tr>
<tr>
<td></td>
<td>1% w/v SDS pH 7.5</td>
</tr>
</tbody>
</table>
7.4.4. Small scale preparation of RNA from *M. xanthus*

This was carried out according to manufacturer’s instructions using the RNAeasy kit from Quiagen.

7.4.5. Ethanol precipitation

DNA pellets were washed with 70% ethanol and centrifuged at 1000rpm/3.50g in a microcentrifuge for 5 minutes. The ethanol was removed and the pellets allowed to dry by vacuum desiccation.

7.4.6. Primer extension analysis

7.4.6.1. End labelling of primer

6ng of the reverse primer was added to 1μl of 0.1M DTT, 2μl of kinase buffer, 2μl of $^{32}$PγATP and 2μl of T4 DNA kinase, 10 μl of water was added and the solution was incubated at 37°C for 1 hour. The reaction was stopped by heating at 60°C for 15 minutes.
The following reagents were added to a 0.5ml Eppendorf tube, 2μl of the 5' end labelled product, 6μl of RNA and 2μl of hybridisation buffer. This was mixed and placed at 80°C for 10 minutes and then at 55°C for 5 minutes to allow annealing. 80μl of fresh primer extension mix and 0.5μl of reverse transcriptase were then added and the mixture was placed at 42°C for 1 hour. 10μl of Sodium hydroxide (1M) was added and the reaction was heated in a boiling water bath for 5 minutes, 10μl of hydrochloric acid was then added with 7μl of 3M sodium acetate (pH 6.3) and 100μl of Derbyshire’s reagent (saturated phenol) (Hodgson pers communication). This was mixed and centrifuged for 5 minutes the aqueous layer was transferred to a fresh tube and the DNA precipitated by the addition of 200μl of ethanol. A pellet was obtained by centrifugation at 1000rpm for 5 minutes. The DNA was ethanol washed, air dried and resuspended in 4μl of formamide dye and 6μl of water. Before loading, the reactions were heated at 80°C for 15 minutes. Samples were then loaded onto a 6% TBE gradient polyacrylamide gel and run at 30 watts for 2.5 hours. The glass plates were removed and the gel was transferred to 3mm filter paper.

7.4.6.3. Materials required for primer extension

<table>
<thead>
<tr>
<th>Primer extension mix</th>
<th>Hybridisation buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M dATP, dCTP, dGTP, dTTP.</td>
<td>2M Sodium chloride</td>
</tr>
<tr>
<td>100M Tris-Hcl pH8</td>
<td>50M PIPES pH 6.4</td>
</tr>
<tr>
<td>10mM DTT</td>
<td>12mM Magnesium chloride</td>
</tr>
</tbody>
</table>
7.4.6.4. Polyacrylamide gels

50x20cm, 0.4mm thick, 6\% polyacrylamide gels were used. A 6\% gel mix was added to two beakers. Polymerisation was engaged by the addition of 2/1000 volume of both 25\% ammonium persulphate and TEMED. 2mls of a 5\% mix was added to the gel initially to form a plug at the bottom of the gel. Then 7mls of the 0.5\% mix was dispersed down the gel plates rapidly to avoid air bubble formation. The plates were lowered to an angle of 20° so that the 5cm wide stream remained down the length of one side. The remaining mixture was added. A gel comb was inserted to a depth of 5mm to form the wells. Polymerisation occurred after 10-15 minutes.

7.4.6.5. Materials required for polyacrylamide gels

<table>
<thead>
<tr>
<th>0.5x TBE 6% gel mix</th>
<th>5x TBE 6% gel mix</th>
</tr>
</thead>
<tbody>
<tr>
<td>75mls 40% acrylamide</td>
<td>30mls 40% acrylamide</td>
</tr>
<tr>
<td>25mls TBE 10x</td>
<td>100mls TBE 10x</td>
</tr>
<tr>
<td>upto 500ml with water</td>
<td>upto 200ml with water</td>
</tr>
</tbody>
</table>

40\% Acrylamide

380g acrylamide
20g NN-methylenbisacrylamide
upto 1 litre with water
7.4.6.6. Primers for primer extension analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position of Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGCGAAGAGGTCTCTGAAGG</td>
<td>carRrev</td>
</tr>
<tr>
<td>CGGAAACACTTTTCGCAGGTGG</td>
<td>carQrev</td>
</tr>
<tr>
<td>CCACCCAGTCCCGACGAAGC</td>
<td>lacZrev</td>
</tr>
<tr>
<td>GTCAGCACCAGGCCACCCACC</td>
<td>gufBrev</td>
</tr>
</tbody>
</table>

Position of primers is shown in Figure 7.1.

![Figure 7-1: Position of primers used in primer extension analysis and RTPCR](image)

Black arrows represent position of primers used in primer extension analysis. Purple arrows represent position of primers used in RTPCR. The primer gufBrev was used in both primer extension analysis and RTPCR.
7.4.7. RT–PCR

Reactions were performed in an eppendorf mastercycler gradient PCR machine and involved an initial incubation at 25°C for 10 minutes followed by a 60 minute incubation at 42°C. To denature the reverse transcriptase, the tubes were heated at 95°C for 5 minutes and then cooled on ice. PCR was performed as stated below.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>15-30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>30-60 seconds</td>
</tr>
<tr>
<td>Elongation</td>
<td>72°C</td>
<td>45secs-3minutes</td>
</tr>
<tr>
<td>Final elongation</td>
<td>72°C</td>
<td>7 minutes</td>
</tr>
</tbody>
</table>

7.4.7.1. Materials required for RT-PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume/sample</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction buffer 10x</td>
<td>2.0μl</td>
<td>1x</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>4.0μl</td>
<td>5mM</td>
</tr>
<tr>
<td>Deoxynucleotide mix</td>
<td>2.0μl</td>
<td>1mM</td>
</tr>
<tr>
<td>Primer</td>
<td>2.0μl</td>
<td>0.75-1.0μM</td>
</tr>
<tr>
<td>Reverse transcriptase</td>
<td>0.8μl</td>
<td>20 units/reaction</td>
</tr>
<tr>
<td>RNA sample</td>
<td>2.0μl</td>
<td>1μg</td>
</tr>
<tr>
<td>Sterile water</td>
<td>7.2μl</td>
<td>-</td>
</tr>
<tr>
<td>Total volume</td>
<td>20μl</td>
<td>-</td>
</tr>
</tbody>
</table>
### 7.4.7.2. Primers for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGGGGCCGCTGCGGCGAAGG</td>
<td><em>crtIP</em>‡1</td>
</tr>
<tr>
<td>GCGCCACTGCATGGCGGGCGG</td>
<td><em>olpA</em> rev</td>
</tr>
<tr>
<td>GGGCGAAGCTGGCATAGTTGC</td>
<td><em>olpA</em> for</td>
</tr>
<tr>
<td>GTCAGCACCAGGCCACACC</td>
<td><em>gufB</em> rev</td>
</tr>
</tbody>
</table>

Position of primers is shown in Figure 7.1.
PCR was performed in a 50μl mixture in an Eppendorf mastercycler gradient PCR machine. Template DNA varied and was either 1μl of a 100x dilution of a previous PCR product, 1μl of a 10x dilution of a plasmid preparation or 5μl of a 100μl solution containing a single colony picked from a plate and resuspended in water. Program A was used to amplify DNA using primers 1 and 2 while program B was used to amplify DNA using primers multi 1, multi 2 and multi 3. A further program, program C was used as the basis for all other general amplifications. Program A involved a hot start, drop down approach with initial denaturation at 94°C. Annealing temperatures were reduced from 72°C - 62°C at a rate of 0.5°C per cycle. This was followed by 10 cycles with an annealing temperature of 62°C. Each cycle also consisted of a 30 second extension step and a final extension step at 72°C for 5 minutes. Program B was also a hot start with 30 cycles and a final extension step as described above, but a constant annealing temperature of 58°C was maintained. Program C also involved a hot start and a 5 minute extension step at 72°C, but with 24 cycles and variable annealing temperatures depending on the TM of the primers. A slight variation on this involved a step up with a low initial annealing temperature for 15 cycles followed by an increase in temperature for a further 15 cycles. The denaturation and extension temperatures remained the same.
### 7.4.8.1. Materials required for PCR

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume 1X</th>
<th>Volume 9X</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction buffer 10X (Invitrogen life technologies)</td>
<td>5µl</td>
<td>45µl</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>2µl</td>
<td>10µl</td>
</tr>
<tr>
<td>Deoxynucleotide mix (1mM)</td>
<td>0.5µl</td>
<td>4.5µl</td>
</tr>
<tr>
<td>Primer (0.75-1.0µM)</td>
<td>0.3µl</td>
<td>2.7µl</td>
</tr>
<tr>
<td>DNA template (1µg)</td>
<td>1µl</td>
<td>9µl</td>
</tr>
<tr>
<td>Water</td>
<td>15.6µl</td>
<td>140.4µl</td>
</tr>
<tr>
<td>DMSO</td>
<td>25µl</td>
<td>225µl</td>
</tr>
<tr>
<td>Taq polymerase (Invitrogen life technologies)</td>
<td>0.3µl</td>
<td>2.7µl</td>
</tr>
<tr>
<td>Total</td>
<td>50µl</td>
<td>50µl (per reaction)</td>
</tr>
</tbody>
</table>

### 7.4.8.2. Primers

Primers were obtained from Invitrogen life technologies and stored at -20°C as a 100pmol/ml solution.
### Primer Sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position of Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>gcgtccgaggtgcctccg</td>
<td><em>carQRS</em> promoter (primer 1)</td>
</tr>
<tr>
<td>cgatcgggtcggggctct</td>
<td><em>lacZ</em> gene (primer 2)</td>
</tr>
<tr>
<td>cggactcaagatgcctgc</td>
<td>Multi 1 <em>attP</em> region</td>
</tr>
<tr>
<td>gactctctggctgggtg</td>
<td>Multi 2 <em>attP</em> region</td>
</tr>
<tr>
<td>cagctttggacctggcag</td>
<td>Multi 3 <em>attP</em> region</td>
</tr>
<tr>
<td>aaaagatcctccgggctactttctcggcatggggggagge</td>
<td><em>orfY</em> for (<em>carQRS</em> operon)</td>
</tr>
<tr>
<td>aaaagatcctccgggctagaaaattcgctggacgctgg</td>
<td><em>orfY</em> rev (<em>carQRS</em> operon)</td>
</tr>
<tr>
<td>tttttaagattctcactgcggccgtgcctgg</td>
<td><em>crtlin</em> for</td>
</tr>
<tr>
<td>gaggagggatcccgctcctcgggatgtgtgcg</td>
<td><em>crtlin</em> rev</td>
</tr>
</tbody>
</table>

Position of primers is shown in figure 7.2.
A = Primer 1 and primer 2 were used to confirm the presence of the carQRS promoter in the pAEB600 series and pSJB700 series. B = Primers multi 1, multi 2 and multi 3 used to screen for plasmid copy number in the pAEB600 series and the pSJB700 series. C = Primers orfYfor and orfZrev were used to amplify the orfY and orfZ genes. D = Primers crtlintfor and crtlintrev used to amplify an internal fragment from crtl.
7.5. Redirect technology – PCR targeted mutagenesis of *M. xanthus*.

7.5.1. Introduction of pMAR202 into *E. coli* BW25113/pIJ790 (λ-red recombination plasmid) by electroporation.

An overnight culture of *E. coli* BW25113/pIJ790 was inoculated in 10ml of LB containing 25µg/ml of chloramphenicol and grown at 30°C. 100µl of the overnight culture was sub-cultured into 10ml of LB containing 20mM MgSO4 and 25µg/ml of chloramphenicol. The culture was grown to an O.D of 0.6 at 600nm. The cells were recovered by centrifugation at 4000rpm in a Wifug bench centrifuge for 5 minutes and then resuspended in 5ml of ice-cold 10% glycerol. This was repeated twice before the final pellet was resuspended in 100µl of 10% glycerol. 50µl of the cell suspension was mixed with 1-2µl of plasmid DNA (100ng/ml). Electroporation was then carried out in 0.2cm ice-cold electroporation cuvette using a BioRad gene pulser II. This was set to 200Ω, 25µf and 2.5kv. 1ml of ice-cold LB was added to the cells they were then incubated at 30°C for 1hour. The cells were pelleted at 1000rpm in a micro-centrifuge and spread onto LB agar plates seeded with chloramphenicol (25µg/ml) and kanamycin (50µg/ml). These were allowed to grow overnight at 30°C.
7.5.1.1. PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td><code>ggcaccgggcgcagctegcgcggcggcgtacatcttccgaggatccggtgacc</code></td>
<td><code>\Delta olpAfor</code></td>
</tr>
<tr>
<td><code>ttcgtggcgcgtccggcgggtccggtgggcggacctactgtaggcctgcttc</code></td>
<td><code>\Delta olpArev</code></td>
</tr>
<tr>
<td><code>gctggagccggagtcgctcgccgcggtgctgtagctgagctgcttc</code></td>
<td><code>\Delta agufBfor</code></td>
</tr>
<tr>
<td><code>caccggcatgagaagaggcatgctgcactttacctccgggattcctcgacc</code></td>
<td><code>\Delta agufBrevid</code></td>
</tr>
<tr>
<td><code>ggcggggcgggggccagctggcatagttgcgaggcatgtgtaggcctgcttc</code></td>
<td><code>\Delta agufDfor</code></td>
</tr>
<tr>
<td><code>ccgccccgctcgagccggggccaggtgctgcacttcgccgggatccgagacc</code></td>
<td><code>\Delta agufDrev</code></td>
</tr>
</tbody>
</table>

Figure 7.3 shows the position of the primers.

![Figure 7-3: Position of primers for PCR targeted mutagenesis.](image)

Black arrows represent primers designed to incorporate the start and stop codons of the gene to be deleted.
Amplification between forward and reverse primers is expected to generate a band which is 78bp larger than the disruption cassette. This is due to the 2x 39bp 5' primer extensions on the forward and reverse primers the apramycin cassette is 1384bp.

### 7.5.1.2. PCR amplification of the extended resistance cassette

PCR was carried out in a mastercycler gradient PCR machine with the following conditions.

<table>
<thead>
<tr>
<th></th>
<th>Temperature</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>2 minutes</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 seconds</td>
<td>10</td>
</tr>
<tr>
<td>Annealing</td>
<td>50°C</td>
<td>45 seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 seconds</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>45 seconds</td>
<td>15</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>45 seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>90 seconds</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 minutes</td>
<td></td>
</tr>
</tbody>
</table>
7.5.1.3. Materials required for PCR amplification

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primers 100pmol/ml</td>
<td>0.5µl</td>
<td>50pmoles each</td>
</tr>
<tr>
<td>Template DNA (1µg)</td>
<td>0.5µl</td>
<td>50ng</td>
</tr>
<tr>
<td>Buffer 10X (Invitrogen)</td>
<td>5µl</td>
<td>1X</td>
</tr>
<tr>
<td>Deoxynuclotides (10mM)</td>
<td>1µl</td>
<td>50µM</td>
</tr>
<tr>
<td>DMSO (100%)</td>
<td>2.5µl</td>
<td>5%</td>
</tr>
<tr>
<td>Taq Polymerase (Invitrogen)</td>
<td>1µl</td>
<td>2.5 units</td>
</tr>
<tr>
<td>Water</td>
<td>36µl</td>
<td>-</td>
</tr>
<tr>
<td>Total volume</td>
<td>50µl</td>
<td>-</td>
</tr>
</tbody>
</table>

7.5.1.4. PCR targeting of *M. xanthus*

A 10ml culture of LB containing kanamycin (50µg/ml) and chloramphenicol (25µg/ml) and L-arabinose (10mM) was inoculated with 100µl of *E. coli* BW25113/pIJ790/pMAR202. After shaking for 3-4 hours at 200rpm the cells were recovered by centrifugation at 4000rpm/1,000g in a Wifug bench centrifuge for 5 minutes. The pellet was resuspended in 100µl of 10% ice-cold glycerol as described above. 50µl of cells was mixed with 1-2µl of the PCR product. Electroporation was carried out as detailed above. The cells were grown for 1 hour at 37°C and then spread onto LB agar plates containing apramycin (100mg/ml) and kanamycin.
(50mg/ml). The plates were incubated overnight at 37°C to promote the loss of the recombination plasmid pIJ790.

7.5.1.5. Verification of the construct

Plasmid DNA was extracted from *E. coli* using the large scale DNA isolation method described earlier. The plasmid DNA was digested with *Sst*I to generate a 750bp internal fragment. This confirmed the presence of the apramycin cassette. The plasmid pMAR202 was then electroporated into *M. xanthus* as detailed above. Colonies were screened on kanamycin (50mg/ml) and apramycin (100mg/ml).

7.5.2. FLP-mediated excision of the disruption cassette

The generation of a non polar, unmarked in frame deletion is facilitated by the use of FLP-mediated excision. A culture of *E. coli* DH5α/BT340 was grown overnight at 30°C in 10ml of LB with 25μg/ml chloramphenicol. This was diluted and grown to an O.D of 0.6 at 600nm. The cells were pelleted by centrifugation at 4000rpm/1.000g in a Wifug bench centrifuge for 5 minutes and the pellet was resuspended in 10ml of 10% ice-cold glycerol. This was repeated twice. The final pellet was then resuspended in 100μl of 10% ice-cold glycerol. 50μl of the cell suspension was mixed with 1-3μl of plasmid DNA (1μg/ml) (pMAR202). Electroporation was carried out as above. The cells were grown at 30°C for 1 hour and then pelleted and plated on to LB agar with apramycin 100μg/ml and chloramphenicol 25μg/ml. After two days incubation at 30°C single colonies were picked and streaked onto LB agar
with no antibiotics. These were incubated at 42°C to induce expression of the flp recombinase and loss of the plasmid BT340. 20-30 colonies were then streaked onto LB agar plates containing apramycin (100μg/ml) and kanamycin (50μg/ml). These were incubated at 37°C. Apramycin sensitive and kanamycin resistant clones demonstrated the successful loss of the apramycin disruption cassette and the generation of a "scar".

7.6. Sequencing of E.coli DNA

The single base pair and 6bp changes were sequenced to validate the loss or alteration of bases. The plasmids were sequenced once DNA had been prepared using the small scale plasmid preparation. Primers for sequencing were obtained from Gibco and were stored at -20°C. They are described above in section 8.4. Sequencing reactions were performed using the method of Sanger et al (1977). 1.2μg of template DNA was added to 3.2pmol of primer and made up to 6μl with water. Taq cycle sequencing was then performed using an Applied Biosystems 373A sequencer (Alta Bioscience, Birmingham, UK).
8. Appendix

8.1. Construction of pSJB500 and further directed mutagenesis of the carQRS promoter

8.1.1. Introduction

Individual base pair changes were made in the carQRS promoter to further assess which bases were required for sigma factor recognition and subsequent transcription. The Mx8 attB site was shown to have a background level of beta-galactosidase activity (McGowan, 1992), (Figure 8.1) meaning activity from the carQRS promoter was reduced in the wild-type promoter pAEB600 in the Mx8 attB site, due to the high background level of activity. Integration at the Mx8 attB site had obvious advantages it did not result in insertional inactivation of the carQRS genes, allowing the modified carQRS promoter to be assessed in an essentially wild-type background (McGowan, 1992; Berry, 1998; Whitworth, 1999). It also enabled transfer of the carQRS promoter fragments into strains of M. xanthus in which the carQRS operon had been deleted (McGowan, 1992). The frequency of gene conversion was also greatly reduced following site-specific recombination (Li and Shimkets, 1988) making the Mx8 attB site an ideal recombination site. The only problem being the background level of activity and the reduced level of activity from the carQRS promoter, which could be due to the distance along the chromosome between the Mx8 site and the carQ and carR genes. In pDAH217 the plasmid integrates at the carQRS operon and activity is increased from the carQRS promoter (Figure 8.2). It was hoped that by adding the carQ and carR genes onto pAEB130 the level of
activity from the wild-type promoter (pAEB600) would increase to similar levels of activity seen in pDAH217 at the carQRS site.

Figure 8-1: Background promoter activity at the Mx8 attB site (DK101::pSJ103)

Y-axis represents specific activity of beta-galactosidase in units/min/mg protein
X-axis represents time in hours (McGowan, 1992)
Figure 8-2: Activity of the carQRS promoter in the carQRS site DK101::pDAH217

Y-axis represents specific activity of beta-galactosidase in units/min/mg protein

X-axis represents time in hours
8.1.2. Generation of pSJB130

The \textit{carQRS} promoter the \textit{carQ} and \textit{carR} genes were amplified using primers \textit{carQ} forward and \textit{carQ} reverse. Restriction sites were engineered into the primers to allow the product to be cloned into a suitable vector. The plasmid pAEB130 was constructed from an \textit{XbaI} restriction of pSJM103. The plasmid pSJM103 has an Mx8 \textit{attP} site and a PI \textit{inc} region allowing transfer to the \textit{M. xanthus} Mx8 \textit{attB} site. The \textit{XbaI} restriction facilitated the removal of a \textit{BamHI} site from the polylinker in pSJM103 leaving a unique \textit{BamHI} and \textit{EcoRI} site upstream of the \textit{lacZ} gene. This allowed \textit{EcoRI/BamHI} promoter fragments to be cloned in a defined orientation. The \textit{carQ} forward primer had an \textit{NcoI} site while the \textit{carQ} reverse primer had an \textit{XbaI} restriction site introduced. The pAEB130 plasmid and the PCR product were digested with \textit{NcoI} and \textit{XbaI} and ligated. Despite several attempts a successful clone was not generated. This procedure was abandoned due to constant failure.

8.1.3. Construction of the pSJB700 series

To introduce the \textit{carQ} and \textit{carR} genes onto pAEB130 proved difficult so an alternative strategy was utilised. Instead of cloning the \textit{carQ} and \textit{carR} genes, \textit{orfY} and \textit{orfZ} were amplified instead. These are open reading frames downstream of the \textit{carQRS} genes identified by sequence analysis of the \textit{carR} region (McGowan et al., 1993). Neither \textit{orfY} or \textit{orfZ} are essential for growth. This was demonstrated by the incorporation of a 14.5kb deletion in the \textit{carQRS} region. This also proved they had no role in carotenogenesis. Both \textit{orfY} and \textit{orfZ} are transcribed divergently with respect to the \textit{carQ carR} and \textit{carS} genes. Similarity searches using Blast show that
orfY and orfZ have homology to a topoisomerase and an oxireductase respectively. It was hoped that by cloning orfY and orfZ onto a suitable vector the plasmid would recombine into this site in the chromosome next to the carQ and carR genes (Figure 8.3). The plasmid pDAH274 has a lacZ gene and PI inc but no Mx8 attP region. The orfY and orfZ genes were amplified using primers orfY forward and orfZ reverse from the plasmid pDAH192 (Hodgson, 1993). BgIII restriction sites were introduced on both primers. A 1kb product was generated pDAH274 was digested with BgIII and the PCR product was successfully cloned into the plasmid pDAH274 to generate pSJB274.

Single base pair changes in the -35 and -10 region of the carQRS promoter were generated as described in chapter three using PCR site directed mutagenesis. Individual primers were used with single bases altered. The individual base pair mutations are shown in figure 8.4. These were all generated in the plasmid pAEB120. The plasmid pAEB120 was digested with EcoRI to liberate a 200bp PCR product, containing the carQRS promoter. Each individual mutation was cloned into pSJB274 to generate the pSJB700 series. These were electroporated into M. xanthus, orientation was checked by PCR using primer 1 and primer 2, used previously in Chapter 2. The multi 1, multi 2 and multi 3 primers were used to detect plasmid integration at the Mx8 site. Beta-galactosidase assays were then used to assess promoter activity. The wild-type carQRS promoter in pSJB700 failed to generate any beta-galactosidase despite several attempts in DK101. This suggested a potential problem with the lacZ gene.
Figure 8-3: Construction of the pSJB700 series

The *orfY* and *orfZ* genes were amplified using PCR and cloned into pDAH274 using *BgIII* sites introduced on the primers *orfY* forward and *orfY* reverse. This generated pSJB274. Each individual promoter mutation was cloned into pSJB274 to generate the pSJB700 series. The pSJB700 series was transferred to *M. xanthus*, promoter activity was assessed using beta-galactosidase assays.
EcoRI

-176  GAATTCCCGCTGCCGCGTCCGAGGTGCCTCCGCGCCAACACTAACG

-130  GCCTCGCCCTCCAGGGCAGGACGGGATGCTGCTGGCGTTCGCAACC

-84  CCGTGACTTTCCAGAGCTTTCTCTCAACCGAACCTTTTGAAGAAGCGCGAG

GCT          CGGA
GCT          ACT

-38  CCGCCGAAACAACCTTTTCGAGGTCGCCTGGCCCTGGAGTCGGGTGATG

-35  RBS

-10  Met

+9  CCGAGCGCAAACGGACGAGCACCACCTCATGAAACCGCATGAATTCC

*Figure 8-4*: Single base pair changes in the -10 and -35 region of the *carQRS* promoter

The DNA sequence shown is the *EcoRI* fragment of pAEB120 containing the *carQRS* promoter sequence. Changes in the sequence engineered into the promoter by site-directed mutagenesis are shown above the wild-type sequence.
However DK101::pSJB700 generated blue colonies on X-gal IPTG plates suggesting that the $lacZ$ gene was functional. After 48hrs LacZ production was detected from cultures of DK101::pSJB700 which had been illuminated. This was considerably less though than expected compared to DK101::pDAH217 and DK101::pAEB600. It is possible that the reduction in $lacZ$ expression at $orfY$ and $orfZ$ is a position effect.
8.1.4. Conclusion

Introducing the carQRS promoter at orfY and orfZ led to a decrease in activity from the carQRS promoter instead of a desired increase in activity. This could be due to a position effect. The plasmid pSJM103 was generated from pDAH274 and was used successfully to generate deletions in the carQRS promoter to define the minimum promoter region required for activity (McGowan et al., 1993). Varying levels of expression were noted from the carQRS promoter in pSJM103. This suggests that it is a position affect in DK101::pSJB700, which results in reduced levels of activity from the carQRS promoter, as a direct result of recombination at the orfY and orfZ site because pSJM103 was functional at the Mx8 attB site. Future work should address the single base pair changes. This could be done by cloning the promoter fragments into pAEB130 and integrating the plasmid at the Mx8 attB site. This would allow promoter activity to be assessed, with regards to individual base changes.
8.2. Appendix 2: Example of sequence data.
9. References


Cole, S. T., Brosch, R., Parkhill, J. *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. **393** 537-544.


Craig, M. L., Suh, W. C. and Record, M. T., Jnr. (1995) HO- and DNase I probing of Eσ70 RNA polymerase-lambda PR promoter open complexes: Mg2+ binding and
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